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THE

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CHANGES IN THE STAINING CAPACITY OF NUCLEAR COMPONENTS DURING CELL DEGENERATION

MAX ALFERT

Department of Zoology, and its Cancer Research Genetics Laboratory, University of California, Berkeley 4, California

Cell degeneration is often accompanied by distinct nuclear alterations which have been classified into several types (*cf.* Ries-Gersch, 1953) on the basis of microscopically observable structural changes. One of these is nuclear pycnosis: the chromatin condenses into one or several homogeneous masses, which, in microscopic preparations, stand out by virtue of their intense staining capacity. A previous cytochemical comparison of fresh and pycnotic tumor nuclei by Leuchtenberger (1950) has dealt with the quantitative behavior of desoxyribonucleic acid (DNA) and proteins during the degenerative process. More recently, a direct staining method for basic proteins has been developed, based on their ability to combine with acid dye at an elevated pH (Alfert and Geschwind, 1953). Routine surveys of various animal tissues by this procedure disclosed the fact that pycnotic nuclei always stain with great intensity, indicating that basic proteins are not lost, but greatly concentrated during this type of nuclear degeneration.

The present paper is concerned with a detailed cytochemical analysis of this degenerative process in a mammalian ovary where pycnotic nuclei are invariably found in old and atretic follicles. This investigation has revealed a pattern of cytochemical changes different from that previously described for tumor nuclei. The data also lend further support to ideas previously expressed by the author (Alfert, 1952), dealing with the mechanism of changes in methyl green stainability of DNA.

MATERIAL AND METHODS

A comparison between normal nuclei from the cumulus oophorus of young follicles and pycnotic masses of chromatin lining the antrum of old follicles was made on the ovaries of an adult guinea pig. Small pieces of the ovaries were fixed for four hours in Carnoy's acetic alcohol or in Baker's formol-calcium and subsequently washed overnight in 95% alcohol or in running water, respectively; the material was then embedded in paraffin and sectioned at various thicknesses. Four staining reactions were subsequently applied: methyl green and Feulgen staining for DNA, fast green staining for histones, and the Millon reaction for tyrosine. The first three of these reactions were quantitated by microspectrophotometric procedures (for details see Pollister, Himes and Ornstein, 1951), comparing the relative dye-binding capacities of normal and pycnotic chromatin in terms of their Feulgen/methyl green and Feulgen/fast green ratios. Several modifications of the routine photometric technique were used in this case and are herewith described in some detail:

1) Rather than measuring a separate series of nuclei for each of the staining reactions used, these reactions can be applied in sequence to one slide, and the same nuclei can be used over again, for successive series of measurements. This procedure greatly increases the precision of the results since sampling errors among independent series of measurements are excluded. Thus the dye-binding ratios of individual nuclei can be computed and averaged in terms of optical densities, and variations in the amounts of stained substrate need not be taken into account. A multiple staining schedule similar to that proposed by Bloch and Godman (1955a) was employed:

The methyl green reaction according to Pollister and Leuchtenberger (1949) was applied to formalin-fixed sections, previously digested with ribonuclease (0.025% for two hours at 40° C. and at pH 6.8) to remove cytoplasmic methyl green staining induced by the fixation (*cf.* Alfert, 1952); series of normal and pycnotic nuclei were mapped out by means of a camera lucida and drawn at a magnification of 3000 ×; nuclear diameters were measured on the drawings and used for computations of nuclear volumes; microphotometric measurements were then made on nuclear cores comprising 50% of the total nuclear volume (core diameter = 0.6 of average nuclear diameter). Subsequently the coverslip was removed and the slide was hydrolyzed for the Feulgen reaction in 1 N trichloracetic acid (TCA) at 60° C. for 12 minutes (the methyl green is extracted during this hydrolysis). The slide was then treated with Schiff reagent and bleaches prepared by substituting TCA for the HCl ordinarily used, and the same nuclei were remeasured in the manner previously indicated. The coverslip was removed again, and the slide was subjected to treatment with 5% TCA at 90° C. for 12 minutes (leading to complete extraction of DNA and Feulgen dye); finally the slide was stained for histones with fast green at pH 8 according to Alfert and Geschwind (1953) and the same nuclei were measured again.

The TCA modification of the Feulgen reaction was designed by Bloch and Godman (1955a) to prevent loss of histones from the nuclei. Formalin fixation of the tissue is required for the same reason but may affect the methyl green stainability of DNA (Alfert, 1952); an independent series of measurements was therefore made, using methyl green and the Feulgen reaction in succession on the same normal and pycnotic nuclei from material fixed in Carnoy's fluid. The orthodox HCl-Feulgen technique was employed in this case, and the sections were again pretreated with ribonuclease to exclude this step as a variable between the two procedures. Independent evidence indicates, however, that the Feulgen/methyl green ratio of Carnoy-fixed mammalian nuclei is not affected by ribonuclease.

In the formalin series a total of 36 normal and 46 pycnotic nuclei were mapped, and measured three times in succession; in the Carnoy series 21 normal and 20 pycnotics were mapped and measured for methyl green and Feulgen only. Three individual values for normal, and one for pycnotic, formalin-fixed nuclei were omitted from the computation of the Feulgen/fast green ratios. The reason was that these values differed by more than 100 per cent from the mean of the population and fell far outside its range of variation. The nuclei omitted belonged to the lower range of diploids in terms of Feulgen dye and showed no peculiarities with respect to methyl green staining; however, they exhibited very low fast green

TABLE I

Ratios between average optical densities at the different wave-lengths used for measurements of normal and pycnotic nuclei (means ± S.E. of 10 determinations).*

			Normal	Pycnotic	Difference in multiples of its S.E.
Formalin-fixed	Feulgen	$\frac{E_{510}}{E_{485}}$	2.32 ± 0.03	2.45 ± 0.02	3.6
	Methyl green	$\frac{E_{650}}{E_{600}}$	2.52 ± 0.03	2.56 ± 0.02	1.1
	Fast green	$\frac{E_{600}}{E_{560}}$	3.03 ± 0.10	3.15 ± 0.14	0.7
Carnoy-fixed	Feulgen	$\frac{E_{560}}{E_{500}}$	2.04 ± 0.03	2.13 ± 0.03	2.1
	Methyl green	$\frac{E_{650}}{E_{600}}$	2.66 ± 0.02	2.62 ± 0.04	0.9

* Example: Carnoy-fixed, methyl green stained normal nuclei were measured at 650 mμ; pycnotics were measured at 600 mμ and their optical densities were multiplied by the factor 2.66.

staining. Bloch and Godman (1955a) also encountered occasional nuclei which, for reasons presently unknown, give a very weak histone reaction.

2) Comparison of normal and pycnotic nuclei revealed extreme differences in staining intensity, falling far outside the range in which valid absorption measurements in one spectral region can be made. Consequently the relatively less dense normal nuclei were measured using wave-lengths at, or close to, peak absorption, while the pycnotic nuclei were measured at wave-lengths farther away from the absorption peak. The optical densities of the latter were then multiplied by an appropriate conversion factor.

The procedure consisted of the following steps: absorption curves for each type of chromatin, each reaction and each fixative were determined on one or two nuclei. Two appropriate wave-lengths were then chosen and the exact ratio between them was established by measuring samples of ten normal whole nuclei at both wave-lengths. To ascertain the extent of possible shifts in the absorption curves of pycnotic chromatin, these same ratios were also measured on pycnotic nuclei sectioned sufficiently thin to reduce optical densities to a range below 1.0. For this purpose approximately 2 μ paraffin sections of Carnoy fixed material and 0.5 μ sections of formalin-fixed material embedded in methacrylate were used. The results of the calibration measurements are given in Table I.

The differences between these ratios for normal and pycnotic nuclei are numerically small in all cases, even in the one instance where it is statistically highly significant. The differences in stainability between normal and pycnotic chromatin turned out to be much larger than could be accounted for by such small curve shifts. In other words "distributional error" (*cf.* Ornstein, 1952) or other errors giving rise to deviations from Beer's law could not have affected the results appreciably. Serious errors due to the effect of stray light on high optical densities were avoided by the device of measuring normal and pycnotic nuclei at different wave-lengths; all measurements on mapped nuclei fell thereby within the range of 16 to 61 per cent transmission. Our microspectrophotometer, similar in principle to that described by Pollister (1952), was used in conjunction with the monochromator of a Beckman B spectrophotometer with 0.2-mm. slit width. Transmitted light was measured in microscopic images projected onto the cathode of a 1P21 photomultiplier tube.

Carnoy-fixed 3 μ sections of ovary were also stained by the Millon procedure for tyrosine (Pollister and Ris, 1947) for cytological study. One such slide was photographed, subsequently stained by the regular Feulgen procedure and the same area rephotographed for detailed comparison of the distributions of protein and DNA in the same cells. Examples of this comparison are given in Figures 3 a,b, and 4 a,b.

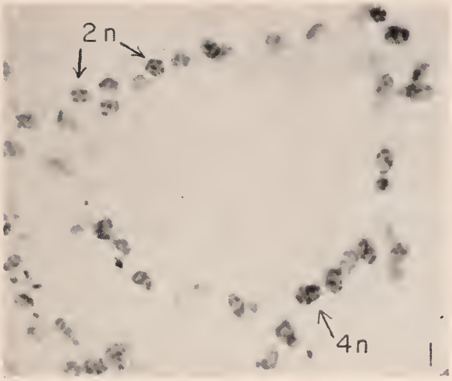
Results

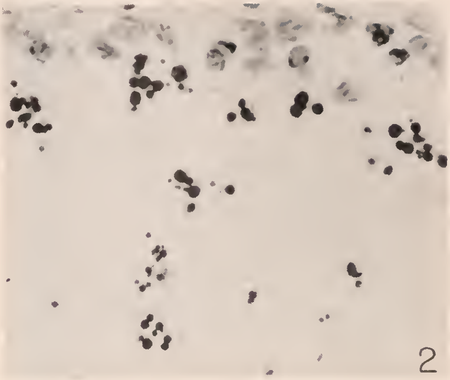
Occasional divisions are seen in normal follicle cells. Using Feulgen-DNA content per nucleus as a measure of the degree of ploidy (*cf.* Swift, 1953) it is obvious that the majority of follicle nuclei are diploid. Occasional tetraploid DNA values probably do not represent permanent tetraploid cells such as those commonly found in mammalian liver, but are pre-prophasic values which one would expect to find in a mitotically active tissue.

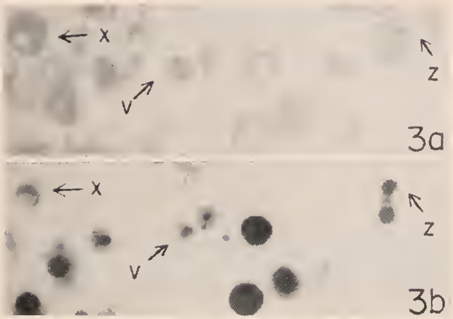
Cell degeneration leading to pycnosis may start in resting diploids as well as in cells with the tetraploid DNA content. The process of pycnosis in this tissue takes a very similar course to that described in Ries-Gersch (1953) and it is almost invariably accompanied by fragmentation of the nuclear material (karyorrhexis): the nuclear substance breaks up into two or several clumps as it condenses, or sometimes into one large clump and a cluster of small granules (see Figures 2, 3b, 4b). Simple photometric determinations can only be done on the larger ones of these masses; in many cases, therefore, not all the nuclear substance of a pycnotic cell was included in the measurement. The appearance of normal and pycnotic cells with the histone reaction is shown in Figures 1 and 2. The Feulgen and methyl green picture is essentially similar, but does not correspond to the appearance obtained by application of the Millon reaction: this can be seen by comparing Figures 3a and 4a to Figures 3b and 4b. The Millon-protein picture shows a great deal of condensation, probably by expulsion of fluid since vacuoles are often encountered. The nucleohistone as visualized by either Feulgen, methyl green or the fast green reaction condenses in an even more striking manner. This process is aptly described as a dissociation ("Entmischung") of the cellular nucleoprotein complexes by Ries-Gersch (1953, page 367).

In Figure 5 individual nuclear DNA contents in arbitrary Feulgen units at peak absorption are plotted against volume, and means and standard errors for normal diploid and pre-prophasic (tetraploid) cells are given. The pycnotic masses have lost volume much more rapidly than DNA content and therefore possess a higher concentration of DNA. As noted by Leuchtenberger (1950) their increased Feulgen density is due to the latter factor and not to any increase in the average amount of DNA. In this particular case the 23 per cent decrease in the average DNA content of pycnotic masses can probably be accounted for by the nuclear fragmentation discussed above, and by the fact that in many instances only part of the nuclear material was of a measurable size. Such fragmentation does not always accompany pycnosis: in autolysing guinea pig kidney slices, many nuclei be-

PLATE I







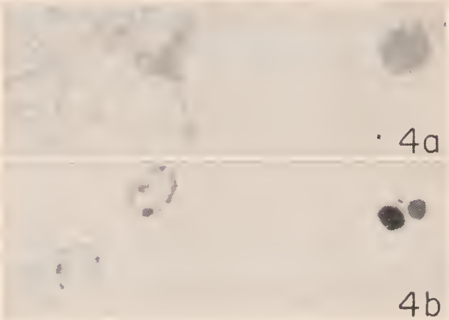


FIGURE 1. Nuclear histone staining (fast green at pH 8) of normal follicle cells surrounding a young oocyte. Formalin, 10 μ, × 570.

FIGURE 2. Nuclear histone staining of pycnotic masses in the antrum of an old follicle. Formalin, 10 μ, × 570.

FIGURES 3 AND 4. Distribution of Millon-protein (a) and Feulgen-DNA (b) in the same follicle cells. Carnoy, 3 μ, × 1100. Figures 3a and b pycnotic nuclei; figures 4a and b normal cells at the left, a degenerating cell at the right. Compare identical nuclei, as identified by arrows, in 3a and 3b. (Photomicrographs by Dr. Walter Plaut.)

come pycnotic by uniform shrinkage without fragmentation and lose none of their DNA during a period of 24 hours autolysis (Roslansky and Alfert, unpublished).

In Table II the Feulgen/fast green and Feulgen/methyl green ratios of the formalin series are given in the first two columns. These data indicate a striking change in the fast green stainability of histone and a small change in the methyl green stainability of DNA. The ratios are lowered, because of a relative increase in acidophilia of histone, and in basophilia of DNA. Put in other terms, there seems to be more histone and more methyl green stainable DNA in the pycnotic nuclei, per unit Feulgen-DNA content, than was present in the normal nuclei from which they were derived.

The methyl green effect is exactly opposite to that described by Leuchtenberger (1950) for pycnotic tumor nuclei: those had an increased Feulgen/methyl green

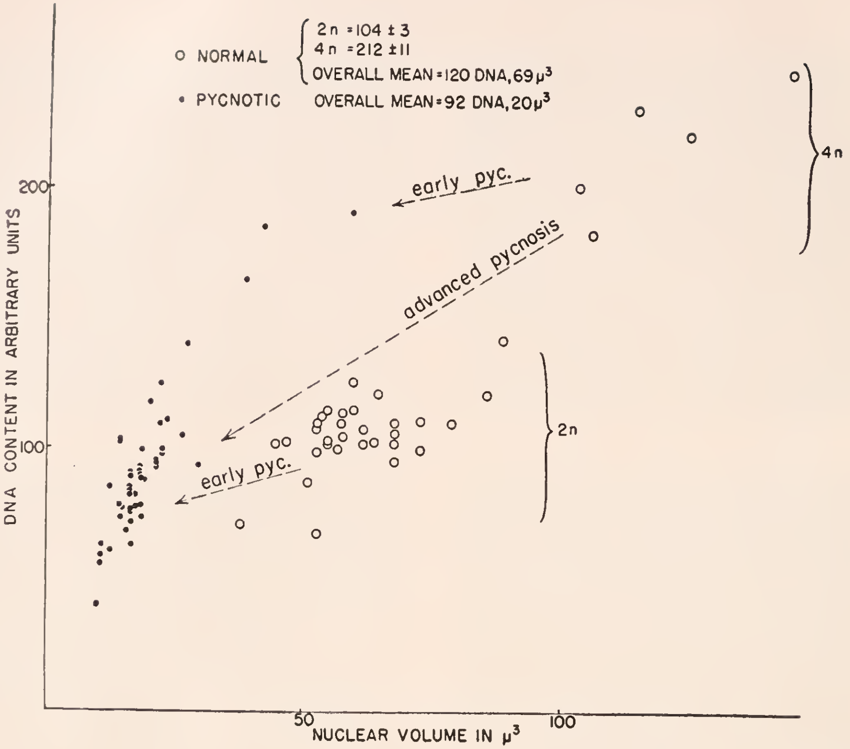


FIGURE 5. The relations between DNA content and volume in normal and pycnotic chromatin of guinea pig follicle cells fixed in formalin.

ratio, *i.e.,* relatively decreased methyl green stainability. In order to see to what extent different types of fixation affect the Feulgen/methyl green ratio, the second series of measurements was performed on nuclei fixed in Carnoy's fluid. These data are presented in the third column of Table II. It was again found that the pycnotic nuclei have a lower Feulgen/methyl green ratio, and that the staining difference was even more pronounced after Carnoy than after formalin fixation. These cases therefore appear to represent a basically different pattern of pycnosis than that described for tumor nuclei.

It is also of interest to compare the difference between the staining capacity of both types of chromatin after Carnoy and formalin fixation in terms of average amounts of dye bound. The compositions of the measured nuclear populations with respect to polyploidy were similar: the formalin data are given in Figure 5; in the Carnoy series 19 diploid nuclei had a mean Feulgen dye content of 55 ± 1.7, and two tetraploids averaged 105; the over-all average was 59, while that of twenty pycnotic masses was 46, a 22 per cent reduction compared to the normals. The complete data for such a comparison are given in Table III.

It can be seen that the Feulgen values of both normal and pycnotic nuclei are about twice as high, after formalin fixation, as they are in Carnoy-fixed material. On the other hand the methyl green contents of pycnotic masses are almost identical

TABLE II

Means ± S.E. of the ratios of optical densities obtained by Feulgen, methyl green and fast green staining. Numbers of determinations in parentheses

	Formalin fixation		Carnoy fixation
	Feulgen* / Fast green***	Feulgen* / Methyl green****	Feulgen* / Methyl green****
Normal nuclei	1.50 ± 0.07 (33)	1.15 ± 0.02 (36)	1.58 ± 0.02 (21)
Pycnotic masses	0.60 ± 0.01 (45)	0.95 ± 0.03 (46)	0.96 ± 0.02 (20)
% change in pycnosis	− 60	− 17	− 39

* Calculated in terms of E_{510}, ~ 51% of peak absorption at 570 mμ.
** Calculated in terms of E_{560}, peak absorption.
*** Calculated in terms of E_{600}, ~ 53% of peak absorption at 635 mμ.
**** Calculated in terms of E_{650}, peak absorption.

after the two types of fixation, while the methyl green stainability of normal nuclei is substantially greater after formalin.

DISCUSSION

The results obtained by the three staining methods employed in this investigation will be discussed separately in the succeeding paragraphs.

a) *The Feulgen reaction*

Measurements of amounts of Feulgen dye were undertaken in order to obtain a standard against which the other two staining reactions could be compared. The average DNA content of pycnotic nuclei (or nuclear fragments) is lower than the average of normal nuclei. The data plotted in Figure 5 clearly show, however, that the largest of the pycnotic nuclei, although they fall into the same size range as normal diploid nuclei, have a markedly higher than diploid DNA content; in all probability these relatively large pycnotic masses result from the degeneration of normal tetraploid cells. The origin of the more frequent small pycnotic masses is less easy to establish: they might either represent early stages of degeneration of normal diploid cells, or more advanced stages of degeneration of tetraploids. The possible origins of the pycnotic nuclei are indicated by the arrows in Figure 5.

TABLE III

Average amounts of DNA in arbitrary units after different fixations

		Carnoy	Formalin	Apparent increase over Carnoy-fixed
DNA content in Feulgen units	Normal	59	120	103%
	Pycnotic	46	92	100%
DNA content in methyl green units	Normal	37	53	43%
	Pycnotic	48	49.5	3%

As indicated above, the degrees of nuclear degeneration in this tissue cannot be easily seriated because karyorrhexis accompanies the degenerative process right from its onset.

The amount of Feulgen dye per nucleus differs by a factor of two in the formalin and Carnoy series. It has been previously known that Feulgen intensity can vary greatly, depending on the type of fixative used, and the conditions under which the reaction is performed (Sibatani and Naora, 1952). These factors have been discussed by Swift (1955). It must again be emphasized that these differences in Feulgen intensity are due to intrinsic factors related to the availability of aldehyde groups for the Schiff reagent, and cannot be explained on the basis of the "distributional error" in cytophotometry (*cf.* Ornstein, 1952). Although the absorption curves of Feulgen-stained nuclei differ somewhat after Carnoy and formalin fixation (the absorption maxima being located at 560 mμ and 570 mμ, respectively), this difference, or any other slight distortion of the curve, is far too small to account for a 100 per cent difference in staining intensity. In the present context it is important to note that, whatever factor might be responsible for the staining difference between the Carnoy and formalin series, it has affected both normal and pycnotic nuclei to the same extent (see Table III).

b) *Methyl green staining of DNA*

The Feulgen measurements after either fixation demonstrated that the pycnotic masses averaged *22–23* per cent less DNA than the normal follicle nuclei. Any decrease in the Feulgen/methyl green ratio indicates a relative increase in basophilia of DNA. Accordingly, each unit of DNA in pycnotic nuclei is able to bind more methyl green than the same quantity of DNA in normal nuclei. This effect is difficult to explain on the basis of the hypothesis, proposed by Kurnick (1950), that methyl green combines only with highly polymerized DNA. However, in the present writer's opinion, there exists little evidence for such a specificity of methyl green when used in ordinary histological procedures. The reasons for this opinion were given in detail in a previous paper (Alfert, 1952). Observations made at that time were in good agreement with the present data and supply a reasonable explanation for the increased basophilia of pycnotic chromatin.

It was formerly noted that intensity of methyl green staining depended on the degree of protein interference, *i.e.*, competition of basic groups of certain nuclear proteins with dye molecules for nucleic acid phosphate groups. Agents which abolished or combined with protein amino groups (acetylation, Van Slyke reaction, formalin treatment) at the same time enhanced the methyl green stainability of DNA. In the former experiments acetylation or Van Slyke reaction had raised the methyl green stainability of Carnoy-fixed mouse pancreas nuclei between 40 per cent and 61 per cent (Alfert, 1952; Table III, p. 153); in the present series (Table III) the dye-binding capacity of normal follicle nuclei is 43 per cent greater after formalin than after Carnoy. It therefore appears that methyl green stainability is partially inhibited in normal follicle nuclei by protein interference, and that most of this inhibition can be removed by formalin treatment. The pycnotic degeneration may by itself have resulted in a physical separation of the nucleoprotein complexes, leading to an unmasking of stainable groups of DNA. Consequently formalin treatment would have no further effect on the dye-binding capacity of pycnotic chromatin. As the data in Table III indicate, almost identical methyl green values were obtained for pycnotic nuclei after either fixation.

This hypothesis can be put somewhat differently in terms of the Feulgen/methyl green ratios listed in Table II. (One can then make the comparison *within* each series of measurements instead of *between* two series of independent samples.) After Carnoy fixation there is a large difference between the Feulgen/methyl green ratios of normal and pycnotic nuclei, because methyl green staining is partially inhibited in the former and uninhibited in the latter. This difference is much smaller in the formalin-fixed series, because formalin had the effect of "equalizing" the staining capacities of normal and pycnotic chromatin by removing at least a large fraction of the staining inhibition present in normal nuclei only.

The protein fractions responsible for staining inhibition, and which may be dissociated and perhaps lost during pycnosis, seem to consist of non-histone proteins. It is known from the work of Mirsky and Ris (1951) that a variable fraction of nuclear DNA may be associated with non-histone protein. Such a condition could be reflected in differences between the Feulgen/methyl green ratios of different nuclear types if one assumes that methyl green cannot displace non-histone proteins, but is capable of competing with histones for binding sites on the DNA molecule. The present fast green data, still to be discussed, justify at least the second part of the foregoing assumption.

In the previous investigation (Alfert, 1952) Feulgen/methyl green ratios of mouse tissues fixed in Carnoy's fluid were determined at different wave-lengths from the ones used here for guinea pig follicle cells. If the data are recalculated for the same wave-lengths, reasonable agreement is obtained: in terms of peak extinctions, the formerly found ratio of 2.47 becomes 1.78 against the present one of 1.58 (Table II). In mouse embryonic nuclei (Swift, 1953) and plant nuclei (Alfert, 1952) very different Feulgen/methyl green ratios have been observed.

c) Fast green staining of basic proteins

The changes in fast green stainability that occur in pycnosis are of the same type as those discussed in connection with methyl green, but more extreme: per unit of DNA the basic proteins bind more dye in pycnotic than in normal nuclei. Basic proteins have thus not been lost, but appear to have become highly concentrated, and even increased, during pycnosis.

In case of the methyl green stainability of DNA, the observed increase in dye binding of pycnotic chromatin could safely be attributed to a mere increase in basophilia, since the concurrent Feulgen measurements demonstrated that the actual amounts of DNA per nucleus had not increased. The fast green measurements are more difficult to interpret. The decreased Feulgen/fast green ratio could have one of two reasons (or a combination of both): 1) either the actual *amounts* of stainable groups have increased in pycnotic nuclei, or 2) the amounts have not changed but their ability to bind acid dye has increased (similar to the increased basophilia of DNA). Until an independent method for the determination of basic proteins in these nuclei becomes available it will not be possible to distinguish with certainty between these alternatives. An actual increase in the amounts of fast green stainable groups could occur by degradation of non-histone proteins into acid and basic residues and subsequent loss of the acid portions only. At present there is no evidence that this actually occurs, but until it is excluded the explanations for the observed change in fast green stainability must of necessity be speculative.[1]

In previous applications of the fast green method for histones it was found that nuclei can undergo wide physiological variations in size and protein content without apparent change in the number of stainable histone groups (*cf.* Alfert and Geschwind, 1953; Alfert, Bern and Kahn, 1955). There are, however, differences in the relative histone stainability among different nuclear types; sperm cells, especially, which are known to contain very basic proteins, stain correspondingly stronger than somatic nuclei. Staining artifacts, due to interference by acid protein, have also been described by Bloch and Godman (1955a) in model systems designed to test the fast green-histone procedure. More recently, Bloch and Godman (1955b) have observed parallel deviations of Feulgen/methyl green and Feulgen/fast green ratios during "differentiation" of formerly rapidly dividing embryonic cells. In that case these ratios increased together as the nuclei grew in size and protein content; in the present case, both ratios dropped as the nuclei condensed. However, changes in these ratios do not always occur simultaneously: the macro- and micronuclei of the ciliate *Tetrahymena pyriformis* have identical Feulgen/methyl green but different Feulgen/fast green ratios (Alfert and Goldstein, 1955). At present it is impossible to decide in which of these instances actual changes in the amount or character of the basic proteins occur, and which result from variable degrees of staining interference by non-histone protein fractions.

CONCLUSIONS

Acid and basic staining methods which depend on salt formation between dye and substrate ions are at least under certain conditions subject to artifacts due to competing ions (*cf.* Swift, 1953). There is strong evidence that such a mechanism is responsible for the difference in methyl green stainability between normal and pycnotic chromatin. The model experiments of Bloch and Godman (1955a) demonstrate that the same effect could also be responsible for the change in fast green stainability of histone. A hypothesis which agrees with the known facts, but which will require eventual analytical confirmation, can be construed as follows:

In normal chromatin DNA, histones and non-histone proteins are intimately combined (*cf.* Mirsky and Ris, 1951; Bernstein and Mazia, 1953). The nature of this complex is such that only a fraction of the acid groups of DNA and of the basic groups of histones are available for dye binding. The pycnotic degeneration leads to a dissociation of this complex that is reflected in the structural changes which accompany pycnosis: nucleohistone condenses to a greater extent than the remaining protein fractions. This dissociation leads to the unmasking of stainable groups in both moieties of the nucleohistone. A similar mechanism has previously been invoked by Kelley (1939) who observed differences in toluidine blue basophilia among resting, dividing and necrotic nuclei and with whose observations the present data agree.

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[1] This possibility was subsequently tested by comparing histone content after pycnosis in two types of nuclei which, in the normal condition, had been found to contain equal amounts of histones but greatly different amounts of total protein. Mouse kidney slices were allowed to autolyse and histones were measured in pycnotic nuclei of collecting ducts and of proximal convoluted tubules. Both types of nuclei exhibited identical increases in histone stainability over the normal condition.

The main interest of the data presented here lies in the possibility that they may furnish an indirect clue to some physical properties of nucleoprotein complexes in normal nuclei, and provide a starting point for further investigations. The apparent existence of widely different patterns of pycnotic degeneration is also noteworthy.

The author received financial support from the University of California Cancer Funds and technical assistance from Mrs. Norma O. Goldstein. We are indebted to Dr. Dorothy Pitelka and Miss Jane Westfall for methacrylate embedding and thin sectioning of some of the material used in this investigation.

SUMMARY

1. Quantitative changes in the staining capacity of nuclear DNA and basic proteins during pycnosis of guinea pig follicle cells were investigated by photometric techniques. The great density of these pycnotic nuclei requires special precautions during photometric analysis.

2. The results show that amounts of measurable DNA, in terms of Feulgen dye, decrease during pycnosis, probably because of progressive nuclear fragmentation. At the same time the methyl green basophilia, per unit Feulgen-DNA, is greatly enhanced. Basic proteins show an even greater relative increase in their capacity to bind acid dye during pycnotic degeneration.

3. These data are interpreted to indicate that a dissociation of the normal nucleoprotein complexes occurs during the degenerative process; this leads to unmasking of charged groups which had previously been unavailable for combination with dye ions.

4. The pattern of pycnosis in the present material appears to differ from that previously described for tumor nuclei.

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IODINE EXCHANGE IN ASCOPHYLLUM

NORMAN A. BAILY[1, 4] AND SALLY KELLY[2, 3]

Radiological Research Laboratory, Columbia University and Marine Biological Laboratory, Woods Hole, Massachusetts

In a preliminary study of iodine uptake in the brown algae (Kelly and Baily, 1951) the process was interpreted as involving an exchange between iodine in the cells and iodine in the sea water. The experiments described here were carried out to verify, elucidate and provide quantitative information on this process. In general, experimental data were obtained by measuring the radioactivity of segments of the brown alga, *Ascophyllum nodosum* (Linn.) LeJolis, and of solutions containing radioactive iodine to which the segments were exposed.

The experimental techniques followed those reported previously with the following exceptions:

1. The medium used as carrier for radioactive iodine was natural sea water rather than Van't Hoff artificial sea water plus iodide. The percentage of radioactive iodine removed from the natural sea water follows closely that from artificial sea water.
2. The liquid samples removed for counting were five ml. rather than two ml. in volume. The larger volume was used to reduce the variations in counting that result from pipetting errors.
3. The amount of radioactive iodine in solution was reduced from 14 to 0.1 microcuries per ml. since a more sensitive Geiger counter was used for sample counting.
4. By means of glycyl-glycine buffer, the pH of solutions during experimental periods was kept at eight to eliminate loss of iodine that occurs at lower pH.

To determine whether the exchange takes place only between living cells and sea water, the removal of radioactive iodine from solution by segments which had been killed by boiling and were no longer respiring was compared with that removed by living segments. After exposure to radioactive iodine for 1.6 hours, the living segments contained considerable radioactivity and the dead ones a negligible amount (Table I). The exchange, therefore, is characteristic of living cells only. This agrees with the previous finding (Kelly, 1953) that uptake of radioactive iodine is decreased by respiratory inhibitors.

To determine whether diffusion, a phenomenon occurring in non-living as well as in living systems, contributed to radioactive iodine removal from sea water

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[1] Address: Roswell Park Memorial Institute, Buffalo, New York.

[2] Address: Division of Laboratories and Research, New York State Dept. of Health, Albany, New York.

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[4] This document is based on work performed under contract AT-30-1-Gen-70 for the Atomic Energy Commission.

TABLE I

Removal of radioactive iodine by living segments and by segments killed by boiling

Exposure period (hours)	Counts per second in segments					
	Living			Dead		
	1	2	3	1	2	3
1.6	87.8	96.8	84.4	1.8	1.8	1.8
14				3.5	3.5	3.5

during long exposure periods, the dead segments described above were replaced in their solutions and allowed to remain for fourteen hours. After this period, their radioactivity had increased only slightly (Table I). Diffusion into segments, then, does not materially contribute to the removal of radioactivity from solutions.

The form in which iodine is removed from sea water is of importance in elucidating the exchange process: iodides and iodates both occur in natural sea water. Removal of radioactive iodine was determined, therefore, in the presence of iodates only and in the presence of iodides only. A solution containing iodates only was obtained through conversion of iodides present in sea water (including added radioactive iodide) into iodates by oxidation with bromine water, followed by boiling to remove excess bromine. As seen in Table II, radioactive iodine removal by segments from solutions so treated is negligible. Exposure of segments for longer periods does not materially increase removal of iodate (Fig. 1). While an exponential uptake is indicated by this curve, the actual amount taken up by the algae is negligible when compared to our earlier results or to those which will be discussed in later sections of this paper. Iodine, then, is not removed from sea water in the form of iodates by this particular system.

A solution containing iodides only was obtained by reducing natural sea water (containing added radioactive iodide) with sodium bisulfite. The pH was adjusted to eight in order to prevent oxidation and iodine loss. The removal of radioactive iodine from such solutions was similar to that from untreated solutions

TABLE II

Removal of radioactive iodine by Ascophyllum segments exposed to solutions containing either iodides only or iodates only. Figures are those from a typical experiment

Exposure period (hours)	Counts per second						
	Solution				Segments		
	0	1	4	24	1	4	24
Iodide	16.3	7.9			100		
Iodate	14.9	14.9			0.8		
	52.2	50.2	49.6	49.3	0.7	0.8	2.1
Natural sea water	135	74.8	66.7	37.7	140	144	178

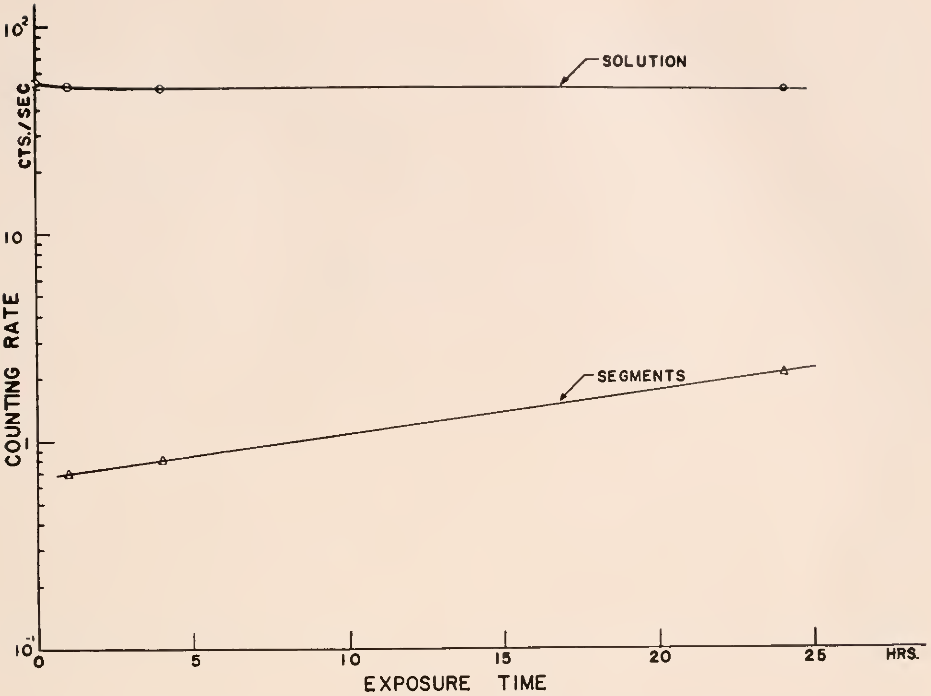

FIGURE 1. Exchange of potassium iodate ions between Ascophyllum and natural sea water containing I^{131}. All iodine in this solution had been oxidized before the Ascophyllum segments were placed in the solution. These points represent the average of three determinations.

(Table II) and far exceeded that from solutions containing iodates only. The iodine removed from sea water by the alga is then in the form of iodides.

The removal of radioactive iodine from sea water over a fifteen- to twenty-hour period follows a definite pattern, interpreted previously as involving an exchange between iodine within the alga and iodine in the surrounding medium. It has further been suggested (Kelly and Baily, 1951) that this exchange process proceeds at two rates: a rapid rate between iodine in the intercellular spaces and the sea water, and a slower rate between iodine in the intercellular spaces and the cells themselves. Indeed, the physical makeup of the alga lends itself to this type of formulation, since the segments are made up largely of loosely packed cells.

The removal of radioactive iodine from sea water (Fig. 2) follows a curve with two distinct slopes. This curve can be obtained mathematically when the following mechanical model is assumed: We visualize a system made up of three separate compartments. The first such compartment is the environment in which the ascophyllum is placed, namely the radioactive sea water. The ascophyllum itself makes up the second and third compartments. These are the intercellular and cellular spaces.

The initial rapid increase of radioactivity in the segments comes about as iodide ions penetrate the outermost layer into the intercellular spaces of the "cortical"

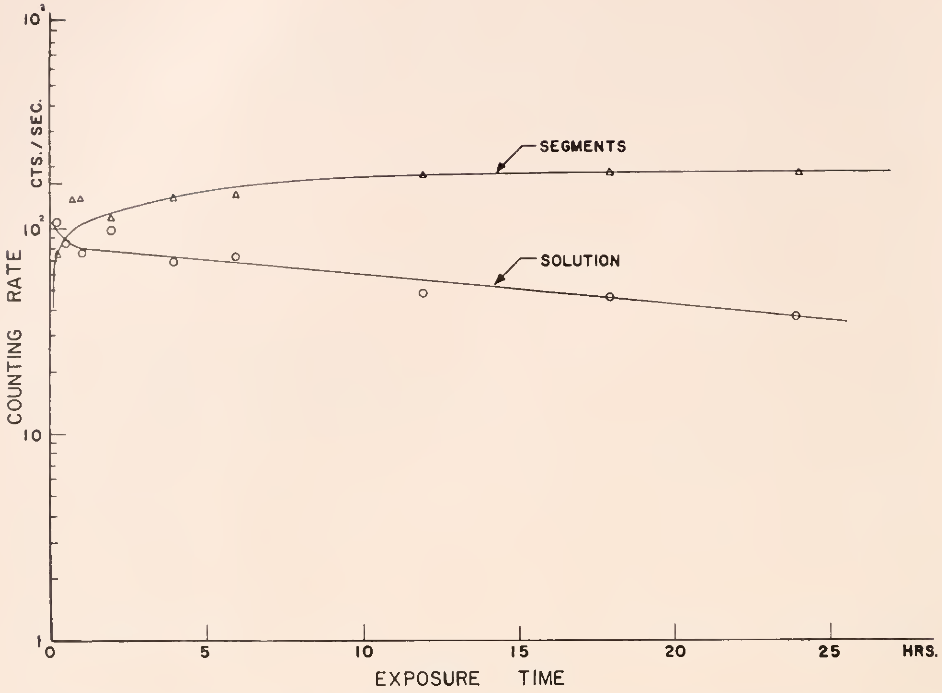

FIGURE 2. Iodine exchange between Ascophyllum segments and a solution of natural sea water containing I^{131}. This curve represents the results of a typical experiment.

region. Here, the entering ions exchange with those present in the intercellular spaces.

Since the ions in the intercellular spaces are readily accessible to the entering ions, the exchange rate between them is rapid. The ions in the intercellular spaces then penetrate the cell membranes and exchange with iodides within the cell. This exchange rate is less rapid, since penetration through membranes must also be achieved.

The initial slope of the curves indicates that the rate at which the iodide ions enter the intercellular spaces is much greater than that with which they enter the cellular space. However, as equilibrium between I^{128} and I^{131} ions in the intercellular space is approached, the slope of these curves approaches that which would be entirely due to the rate of ion transport from intercellular to cellular space. Finally, as the curve becomes truly exponential, its slope will be the rate at which iodide ions are moving from intercellular to cellular space. The model and the experimental evidence suggest, also, that the non-cellular material on the outer cells of the alga is more permeable to the iodide ion than are the cell membranes. The failure of the curve to reach zero slope suggests further that iodine may be used by the cells in metabolic processes. The equilibrium that was reported attained in previous work, was probably not an equilibrium at all but a levelling off of iodine removal because of iodine loss from the solution with time, since no attempt was made in the previous work to control pH.

In preliminary experiments designed to determine the rate of iodine removal, the alga was exposed to sea water containing radioactive iodine for short periods of time, as indicated in Figure 3. While the data obtained could not be used to calculate the actual rate, the curve indicates a total uptake increasing exponentially and consequently an uptake rate which is a function of time.

Since it was found that the exchange process involved iodine which is in the form of iodides, the removal of radioactive iodine was studied in which all the iodine was reduced to iodides. The concentration of iodine in this experiment was 200 gamma per liter. This high concentration of iodide was used since it became evident that the amount of iodine exchanged was dependent upon concentration. Under natural conditions, the amount of iodine exchanged by the alga is limited obviously by the concentration rather than by the total amount of iodine available. This and subsequent experiments show that a concentration of 200 gamma per liter in the volume provided is not sufficient to bring the segments to a saturated condition (Figs. 4 and 5).

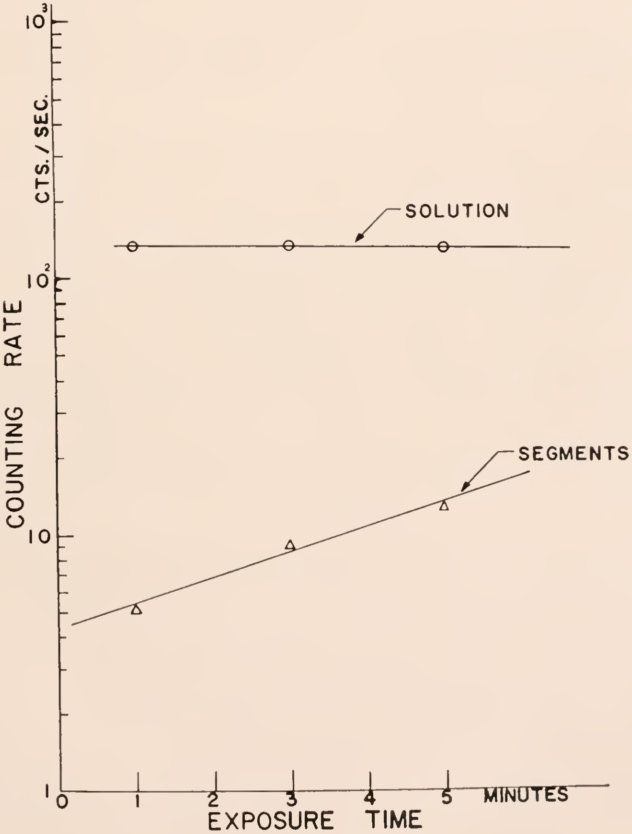

FIGURE 3. Iodine exchange between Ascophyllum segments and natural sea water containing radioactive iodine. Each point is the average of three determinations.

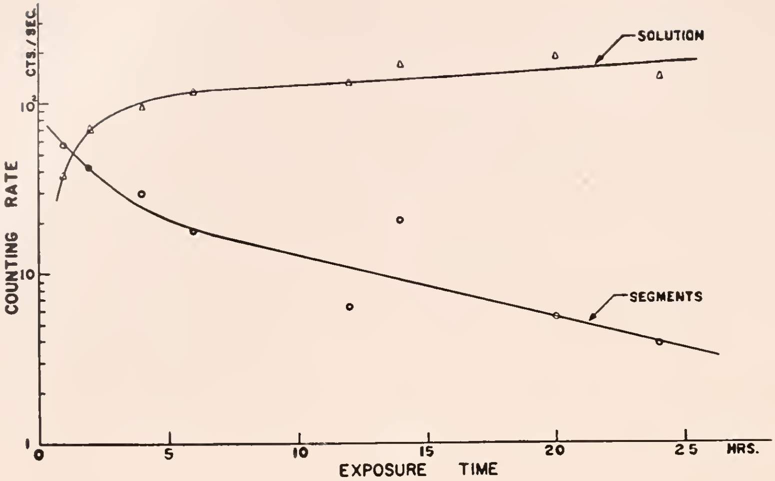

FIGURE 4. Exchange of iodide ions between Ascophyllum and natural sea water having a total iodide content of 200 gamma per liter. All iodine was converted to iodides. Solutions were buffered to pH 8 using glycyl-glycine.

If an exchange of iodine occurs, then segments pre-soaked in radioactive iodine solutions will release their radioactivity to similar but non-radioactive solutions. As indicated in Table IV, the release of radioactive iodine from presoaked segments followed the course which would be expected from an exchange process.

The removal of radioactive iodine from sea water by whole plants, using a volume of 825 ml. per plant, is similar to that removed by groups of segments.

In order to demonstrate that the iodide concentration influences the exchange rate, removal of iodine was studied when the external concentration of iodide was varied. If segment permeability is assumed to remain constant during the experimental period, the only other factor influencing the exchange rate is the relative concentration of iodine in the segments and in the surrounding medium. As the external concentration of iodide is varied, the per cent decrease in radioactivity of

TABLE III

Radioactive iodine removed from solution by Ascophyllum segments as a function of time and of initial iodide concentration. Each figure is the result of three determinations

Concentration of iodide (mg./l.)	Per cent of initial activity remaining in solution Exposure time (hours)		
	1	4	24
0.5	89	69	29
0.7	86	—	38
0.75	89	67	22
2.0	—	77	21
5.0	—	80	27
100	—	93	91

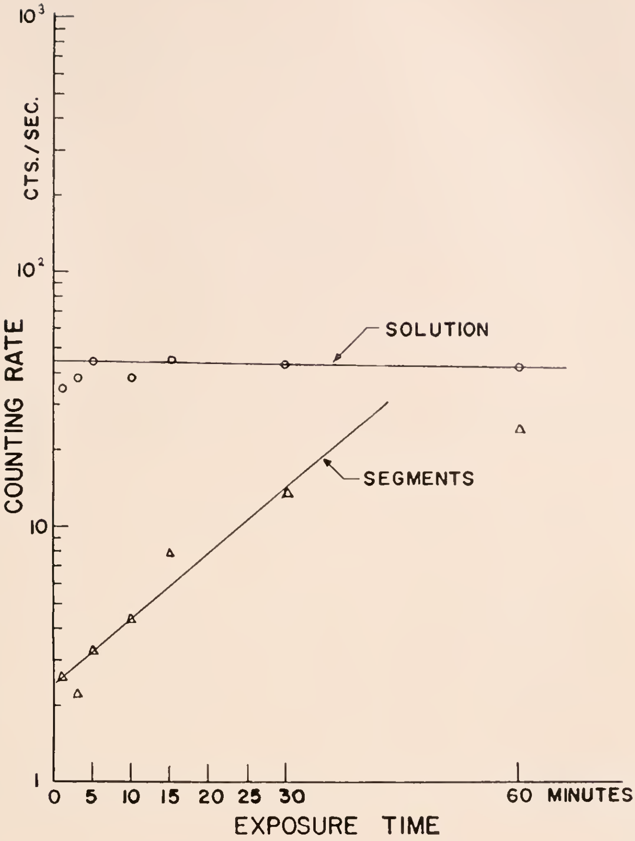

Figure 5. Iodide exchange between Ascophyllum segments and sea water having iodide content of 200 gamma per liter. Each point represents the average of five determinations.

the surrounding medium during a certain time interval should remain constant. As indicated in Table III, the amount of iodine exchanged is dependent upon external concentration for iodide concentrations of less than 5 milligrams per liter of sea water under the experimental conditions employed.

Table III also suggests that even at extremely high iodide concentrations (100 mg./liter) natural conditions are not duplicated. Since the alga has pre-

Table IV

Radioactive iodine exchanged into sea water solutions by segments of algae which had been soaked in radioactive sea water

Time (hours) after radioactive segments were placed in the solution	Counts per second in solution			
	1	4	24	50
	1.2	4.2	11.3	11.6

viously been in an environment capable of supplying all the iodine it was capable of absorbing, it is assumed to be in an iodine-saturated condition. If so, when it is exposed to a radioactive sea water solution, the total amount of radioactivity removed from this solution would not be dependent on iodide concentration provided the concentration is great enough to carry out the exchange process at the same level as would take place in its natural habitat.

Under conditions of saturation, the radioactivity removed from solution should reach a stable value. That is, a given amount dependent on the ratio of I^{128} to I^{131} would be removed, and no further depletion of radioactivity in the solution would take place as time progressed. This amount removed would then tell us the ratio of total iodine content within the alga to that in sea water.

This condition was satisfied experimentally in the following manner: alga segments were transferred hourly to 10-ml. sea water solutions containing one mg./liter iodide, and the decrease in radioactivity of each solution measured. This procedure was repeated until the solutions no longer showed a decrease in radioactivity. Twenty-one to twenty-four hours was the time required for this condition to be reached.

To calculate the ratio of the initial concentrations of iodine in the segments to iodine in the solution, the following procedure was used:

Let I_0 = the amount of I^{128} in the initial solution
f = the fraction of I^{131} in the initial solution
f_h' = the fraction of I^{131} in solution at the end of h hours
I_0' = the amount of I^{128} initially in the segments
f_h = the fraction of I^{131} in the segments at the end of h hours.

Now the amount of I^{131} in solution at $t = 0$ must be exactly equal to the amount of I^{131} in solution at $t = h$ plus the amount of I^{131} in the segments at $t = h$.

$$\therefore \quad fI_0 = f_h' I_0 + f_h I_0'.$$

At the end of n hours, when equilibrium is reached,

$$\left[nf - \sum_{i=1}^{n} f_i' \right] I_0 = f_n I_0'.$$

At equilibrium, $fn = f$,

$$\therefore \quad n - \sum_{i=1}^{n} \frac{f_i'}{f} = \frac{I_0'}{I_0},$$

or,

$$I_0' = \sum_{i=1}^{n} \left[1 - \frac{f_i'}{f} \right].$$

The average ratio (3 determinations) of I_0' to I_0 by this procedure is 4.20 ± 0.16.

The volumes of solutions and segments were 10 ml. and 0.189 ml., respectively. These give a value of 220 for the ratio of the concentrations.

On the basis of the fresh weight of the alga, the amount of iodine contained by the segments is 0.19 milligram per gram.

SUMMARY

1. Through the use of radioactive iodine the following information concerning the removal of iodine from sea water by Ascophyllum has been established: the removal occurs as an exchange process between iodine in the sea water and iodine already present in the cells; the iodine is exchanged in the form of iodides, not iodates; the exchange takes place in living cells only and in entire plants as well as the isolated segments studied in detail.

2. The exchange rate is dependent upon concentration of iodine available. Two different exchange rates may exist, suggesting that a two-step process is involved.

3. The iodine concentration within the alga is about 220 times that of sea water and the absolute iodine content of the alga examined was 0.19 mg./g. fresh weight.

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INTRACELLULAR SYMBIOSIS IN COCKROACHES. I. PRODUCTION OF APOSYMBIOTIC COCKROACHES[1]

MARION A. BROOKS AND A. GLENN RICHARDS

Department of Entomology and Economic Zoology, University of Minnesota, St. Paul 1, Minnesota

Most of the literature on endosymbiosis is of a descriptive rather than an experimental nature, and the theories on the role of the symbiotes are usually based on circumstantial evidence. Practically all symbiote-bearing insects feed on diets which are incomplete or inadequate in certain substances known to be required by non-symbiotic insects or by vertebrates (Buchner, 1953). The only known symbiote-bearing insects among the general feeders are the ants, primitive termites, and cockroaches. Interestingly enough, parasites feeding on vertebrate blood do not possess symbiotes if during their larval stages they feed on a general diet, as is the case with mosquitoes, fleas and tabanids. It is also well known that among the Hemiptera-Homoptera, symbiotes are found only in the bugs which feed on vertebrate blood or plant juice but not in the bugs which are predacious on other insect species.

In any insect species which has intracellular symbiotes, the microorganisms have been found in every individual that was examined for them. Frequently the symbiotes are in anatomical relation to the insect's digestive tract. Thus it seems logical to ascribe to symbiotes a role in the nutrition of the insect host.

Hypotheses as to symbiotic functions are difficult to prove simply because in the majority of cases the two organisms are by nature inseparable. In those cases where the symbiotes are transmitted from one generation to the next as contaminants on the surface of the egg it is relatively easy to obtain aposymbiotic insects.[2] By surface-sterilization of eggs, larvae of a few insects have been obtained free of intestinal symbiotes and these larvae were unable to grow and reproduce normally (Koch, 1933; Schneider, 1940; Wigglesworth, 1952; Fraenkel, 1952). Pant and Fraenkel (1954) have actually identified certain B-vitamins and sterols provided by the yeast symbiotes of two beetles.

But when the symbiotes are intracellular and are transmitted in the cytoplasm of the egg to an intracellular location in the embryo, the sequence is far more difficult to interrupt. The only literature known to the present authors of removal of intracellular symbiotes is the work of Aschner (1934) and Aschner and Ries (1933) on the body louse. In young embryos of this insect, the symbiotes congregate in a stomach disc, and during larval development the symbiotes migrate from the stomach disc to an ovarial mycetome from which they ultimately infect the eggs. If embryos were centrifuged so that the stomach disc, containing the symbiotes, was displaced, the symbiotes were unable to reach the mycetome. Larvae with uninfected mycetomes grew poorly and died prematurely. On the other hand, if infected mycetomes were surgically removed from normal larvae, the symbiotes were unable to reach the ovaries and this was followed by lack of egg development. These experiments indicate that the symbiotes are involved in both growth and reproduction.

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[2] Several entomologists have queried our use of the word "aposymbiotic." It seems desirable to distinguish between insects which normally do not have symbiotes and those which normally do but which have been deprived of the symbiotes. The distinction is expressed by "asymbiotic" in the former case and "aposymbiotic" in the latter.

The cockroach was one of the first insects recognized as having intracellular bodies presumed to be symbiotes (Blochmann, 1887). Blochmann discovered within the abdominal fat body discrete mycetocytes packed full of rod-shaped objects which he called *bacteroids.* Several later authors contributed information on the fine morphology of the bacteroids, the manner of their transmission, and the embryological development of the cockroaches (for reviews and complete bibliography, see Buchner, 1953; Steinhaus, 1947).

According to Buchner (1953), the presence of bacteroids has now been proved in 25 species of 16 genera of cockroaches so far examined. Although the microanatomy and details of transmission and embryological development vary from species to species, the general type of bacteroid-infection holds true throughout the whole order.

In brief, the bacteroids are always restricted to the mycetocytes (Fig. 1) of the fat body in both males and females except that in females some mycetocytes migrate to the ovaries in early nymphal life (Fig. 2). Bacteroids enter the ovarioles but the method of penetration of the tunica propria is unknown. The bacteroids remain in a peripheral layer within each oöcyte as it develops (Fig. 5).

In males, mycetocytes surround the testes (Fig. 6) of young nymphs but bacteroids have not been found in the testes and there is no evidence that bacteroids are transmitted by males.

During embryological development, the bacteroids are carried to the center of the egg with the cleavage nuclei. Although a variety of processes intervene at the next step in different species, in the subsequent development of the German cockroach, at least, the bacteroids get into the already-formed mycetocytes while the fat body is still segmented (Koch, 1949). Here again the method of migration of the bacteroids is unknown, as they give no evidence of motility. In young embryos of the German roach, the mycetocytes differentiate in clusters, one in each lateral half of abdominal segments 2 through 6. As development proceeds, the mycetocytes separate, increase by mitotic divisions (Brooks and Richards, 1955a), and become distributed throughout the visceral fat body of the abdomen. They do not enter the first, seventh, or eighth abdominal segments, the peripheral fat, or the thorax.

The visceral fat body of the abdomen is a diffuse, lobulated or branched tissue filling the hemocoel. It surrounds the intestinal canal and the gonads, and is itself enmeshed with tracheoles and Malpighian tubules.

The mycetocytes are distinctly different from the other cells of the fat body.

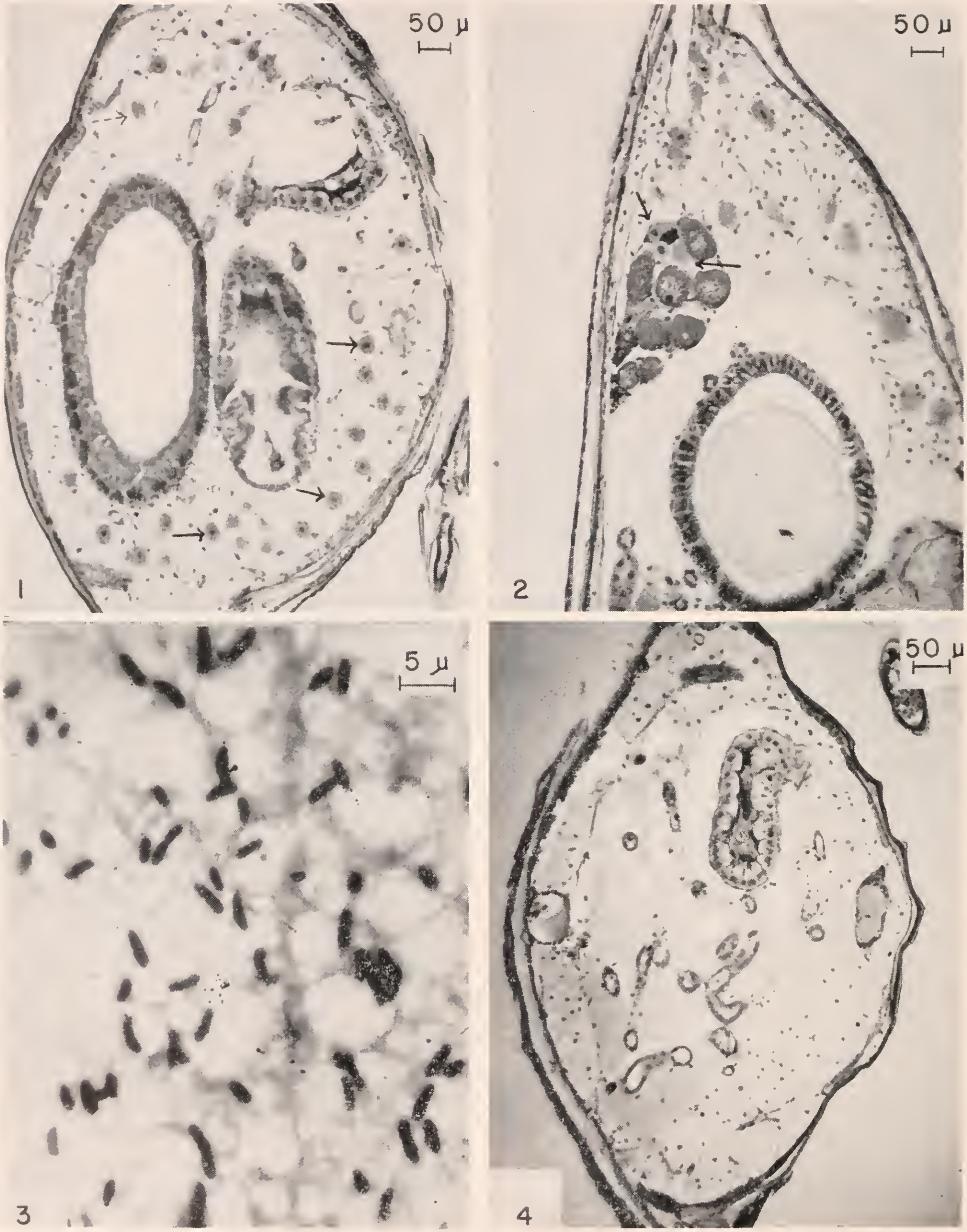


Figure 1. Mycetocytes (several indicated by arrows) in fat body as seen in cross-section of a three-week old German cockroach nymph. The section is turned so that the ventral side of the insect is on the right side of the photograph. Fixed in Carnoy's fluid, sectioned at 10 μ, stained with Delafield's hematoxylin and counterstained with clove oil saturated with erythrosin. After this treatment, the mycetocyte nuclei are blue and the bacteroids *en masse* are rose-violet or red. Individually the bacteroids appear hollow, the cell walls distinct and purple. The section was photographed with the aid of a Wratten red filter A (25).

The mycetocyte nuclei are relatively large and, in thin sections following Giemsa's stain, one may see that the cytoplasm appears fibrous. In normal roaches the mycetocytes are filled with bacteroids and have a sub-spherical or slightly stellate shape, with a diameter in the order of magnitude of 20 μ.

The method of transmission which has been described for cockroaches is called "hereditary transmission" or "transovarial infection."

Blochmann (1887) found that the bacteroids stained positively with Gram's stain. Figure 3 is a photomicrograph of Gram-stained bacteroids liberated from mycetocytes by crushing a piece of fat body on a microscope slide. The bacteroids in German roaches are approximately 3–6 μ in length and 0.9 μ in diameter. They are frequently seen in what appears to be a process of transverse constriction.

Numerous attempts to prove the bacterial nature of the bacteroids by culturing them have at best been ambiguous. Gier (1947) states that the most perplexing problem in symbiote cultivation is the identification of the cultured organism. This is true because there has not been a cockroach positively known to have been deprived of its microorganisms so that a modified statement of Koch's third and fourth postulates could be tested.

As Lederberg (1952) stated (p. 415) in respect to symbiotic problems in general, "Too little emphasis has been placed on the occurrence and behavior of 'disinfected' or aposymbiotic individuals, and on the criterion of re-infection for the specificity and identity of the endosymbiotic microorganism."

Brues and Dunn (1945) attempted to eliminate the bacteroids by injecting various sulfa drugs and penicillin into the large tropical roach, *Blaberus craniifer*. Doses of sulfa comparable to or higher than the mouse tolerance had no effect on the bacteroids. Penicillin, on the other hand, if given in tremendously large doses killed, or at least greatly reduced, the bacteroids (as observed in stained sections); but the cockroaches died. Since death did not result immediately, the authors thought it was caused by the lack of the bacteroids rather than by the toxic effects of the penicillin.

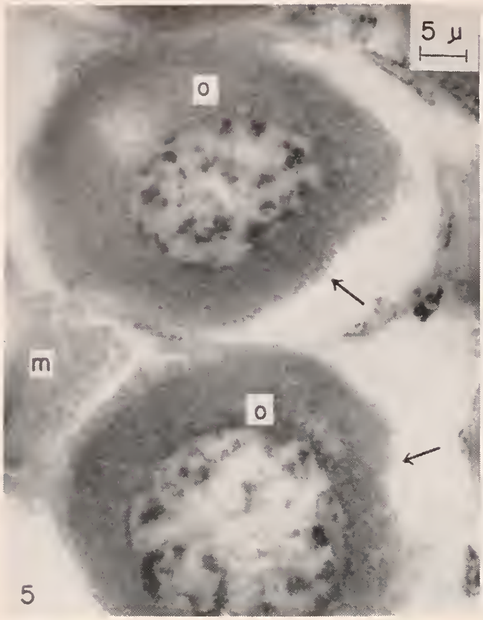
Glaser (1946) administered sodium sulfathiazole in the drinking water and injected sodium and calcium penicillin into the body cavities of adult American roaches, *Periplaneta americana*. He also subjected a few adults and some nymphs to prolonged high temperature (39° C.). About 38% of all his treated animals survived. Upon sacrificing the survivors, he observed that the bacteroids in the fat body and in the ovaries were either modified or absent and that the ovaries themselves had usually retrogressed. Glaser made the interesting observation that heat treatment of juveniles abnormally prolonged their development.

Noland (personal communication) confirmed Glaser's penicillin and heat effects and also extended sulfa treatments to include the German roach, *Blattella germanica*. In every instance where the bacteroids were reduced to the vanishing point, the ovaries were incapable of reproduction. Both Glaser and Noland based their diagnoses on Gram-stained smears.

FIGURE 2. Mycetocytes (indicated by arrows) within the ovary of a three-week old nymph. Prepared as above.

FIGURE 3. A smear of fat body from a German cockroach stained with Gram's stain. Bacteroids are Gram-positive.

FIGURE 4. Cross-section of a young aposymbiotic nymph. There are no mycetocytes or bacteroids in the section. Prepared as in Figure 1.



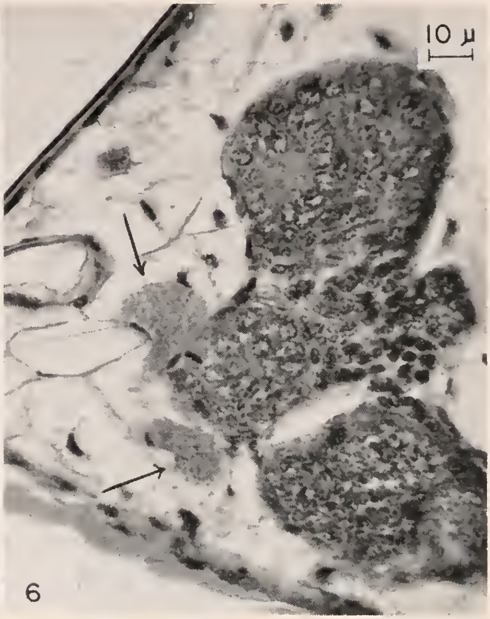


FIGURE 5. Bacteroids (appearing circular in section) forming a peripheral layer under the tunica propria of young oöcytes. m = mycetocyte; o = oöcyte. Prepared as in Figure 1.

FIGURE 6. Mycetocytes (indicated by arrows) appressed to or near the testes of a three-week old nymph. Prepared as in Figure 1.

Conclusive results were not obtained from the series of experiments mentioned above because it was impossible to distinguish the effects of the drugs or heat from the effects of the loss of the bacteroids.

The present study was planned as an extension and elaboration of the works of Brues, Dunn, Glaser, and Noland with the hope that among the newer antibiotics there would be one more effective in eliminating the bacteroids and yet allow the treated cockroaches to live and reproduce.

MATERIALS AND METHODS

The German cockroach, *Blattella germanica* L., was used primarily because of its short life cycle. Another fact in its favor is its small size, which reduces the expense and labor of making serial sections of whole insects. The original colony was established by catching a few adults in the laboratory building. Females with ripe egg capsules were individually segregated in small cages and the nymphs, upon hatching, were randomly divided and distributed to several matched cages for testing antibiotic and dietary variables. In this way groups of 8 to 10 genetically similar insects were used for comparing sets of 4 differently-supplemented diets.

Records were kept of the dates of maturity, the appearance of the first egg capsules, the hatching of the first nymphs, and the death of the individuals of the first generation. The first filial generation was again divided and distributed and similar records kept for them, and in certain cases the second filial generation was studied likewise. In this way pedigrees were established for three-generation spans on some of the diets.

A total of 18 different antibiotic-diet combinations was fed over a period of 15 months. Once the effects of the antibiotics or diets became known, the laborious record-keeping became unnecessary and larger pooled groups were used for obtaining growth curves. The nymphs were anesthetized with carbon dioxide gas, brushed clean of food and debris, and weighed in a vial on an analytical balance.

The crude diet used for feeding stock cultures of the cockroaches and for a base in some of the experimentally supplemented diets was a dog biscuit known as "Morton's Kibbies," obtained from the Morton Dog Food Company of Minneapolis. The content of this biscuit is given on the bag as follows:

corn meal
kelp
baking powder
second clear flour
brewers yeast
fish meal
soybean oil meal
feeding oat meal
linseed oil meal
Fleischmann's irradiated dry yeast
cod liver oil
meat and bone meal
standard wheat middlings
chlorophyllin (in a carrier of dehydrated alfalfa leaf meal)

The analysis of the biscuit is given as follows:

protein, minimum = 19.00%
fat, minimum = 2.50%
fibre, maximum = 4.00%
NaCl, maximum = 1.13%
Ca, minimum = 2.42%
P, minimum = 1.235%

The mineral content, less than 5%, is salt, potassium iodide, iron oxide, manganese sulphate, and calcium carbonate. After the ingredients are mixed, they are toasted at 380–390° F. (= 193–199° C.) for 32 minutes.

When this dog biscuit was used for experimental insects, it was ground in a food grinder and then finely pulverized in a ball mill.

The antibiotic and sulfa-drug supplements were from the following sources: chloromycetin (chloramphenicol), Parke, Davis and Company; aureomycin, crystalline, Lederle, Lot CP-103-1; aureomycin calcium drops, complimentary sample, Lederle Laboratories Division, American Cyanamid Company; sodium sulfathiazole (sesquihydrate) Merck U.S.P., complimentary sample, Merck and Company, Inc.; succinyl sulfathiazole Mann U.S.P., complimentary sample, Mann Fine Chemicals, Inc.

For heat treatments, the cockroaches were subjected to high temperatures of repeated short exposures in a heating chamber patterned after that of Noland (1944) or to somewhat lower temperatures for constant long exposures as Glaser (1946) had done.

Determination of the presence and condition of the bacteroids was made almost entirely by histological serial sections because smears proved unreliable. The material was routinely fixed in Carnoy's fluid, dehydrated through an ethanolbutanol series, embedded in paraffin, sectioned at 10 μ. and stained with Delafield's hematoxylin followed by counter-staining with clove oil saturated with erythrosin. Flemming's fixing fluid and Heidenhain's iron hematoxylin stain were less satisfactory.

In some instances, fresh fat-body tissue or entire excised ovaries were incubated with neotetrazolium chloride by a special technique which stained only the bacteroids (Brooks and Richards, unpublished data).

RESULTS

1. Effect of heat treatments

One brood of newly-hatched German cockroach nymphs was subjected on successive days to a 0.6° C. rise per minute until 40°, 41°, 42°, 43°, and 44° C. were reached on respective days. On the last day only 20 of the 30 insects recovered. Several were sacrificed for histological study and the rest were kept at 25° C. without further heating. Of the 14 survivors, only 5 lived to maturity. They appeared normal and produced offspring.

A second group of nymphs was brought to 40°, 42°, and 42° C. on three alternate days and maintained at those temperatures for 30 minutes. Several of these nymphs were sacrificed for histological sections immediately following the final heat treatment.

The sections from both treatments were practically identical. The heat had caused a gross emaciation of the fat body so that it was only a thin sheath instead of plump lobes. The fat cells were the component which had suffered, as they had lost most of their cytoplasm and were reduced to little more than nuclei and cell membranes. However, the only observable effect on the mycetocytes was that they were compactly rounded instead of stellate. As a net result the fat body consisted chiefly of mycetocytes. Some of the insects which survived the short heat treatments were sectioned 5 weeks later and at that time they presented a normal histological picture.

Constant high temperature of longer duration was then used in five other experiments. The insects were acclimatized to 37° C. at 50% relative humidity, which exceeded the incipient lethal temperature, *i.e.*, the highest temperature beyond which the insects could no longer live for an indefinite time. The effect of this temperature was studied on about 190 newly-hatched nymphs and 55 adults.

Exposing nymphs to 37° C. for 14 days caused the same shrinkage effect of the fat body as had resulted from higher temperatures of shorter duration. The subsequent growth and maturation of the nymphs was delayed slightly.

However, an exposure of between 17 and 22 days killed two-thirds of the nymphs by the time the heating period was ended and destroyed most of the bacteroids in the survivors. At the next molt, the cuticle of the survivors became a golden tan color instead of the normal dark brown-and-black. The subsequent growth of these nymphs was delayed by a period exceeding the length of the heat treatment; and only about one-third of them eventually reached maturity.

Smears made of fat-body biopsies of some of these retarded insects failed to indicate the presence of any bacteroids but subsequent complete sets of serial sections of the same individuals showed normal mycetocytes although their numbers were reduced. It is for reasons such as this that smears are unreliable indicators of the number of bacteroids.

The most adversely affected nymph was sacrificed 35 days after removal from the heat and although every section was examined, no normal mycetocytes were found. But a few bacteroids persisted in occasional mycetocytes.

One pair of cockroaches from this experiment produced offspring. About half of the eggs in the egg capsule did not develop, and of the formed embryos, only two hatched. The mycetocytes in one of these nymphs were not fully developed while those in the second were drastically retarded, containing only a few bacteroids.

Compared to nymphs, recently emerged adults were less resistant to 37° C. Two-thirds of the adults died in 10 days in contrast to 17–22 days for nymphs. The life span of the surviving adults was cut to less than one-third of the normal expectancy. The males were discarded as their sexual organs were badly damaged and normal males were mated to the treated females. Each female laid one abortive egg capsule before she died except for one individual which laid two capsules, nymphs hatching from the second capsule.

These experiments determined that 1) heat destroys some bacteroids; 2) heat treatment of nymphs delays subsequent growth; 3) heat treatment of adults retards reproduction; and 4) even though roaches which had been treated as nymphs seemingly recovered, certain adverse effects were passed on to the next generation. But definite conclusions as to the function of the bacteroids could not be drawn from these experiments because 1) it was impossible to distinguish the deleterious effects of the heat *per se* on both growth and reproduction from the lack of bacteroids; 2) it was impossible under the conditions of the experiments to *completely* eliminate the bacteroids without killing the cockroaches; and 3) the residual bacteroids in surviving insects evidently multiplied and approached a normal population.

2. *Effects of feeding drugs and antibiotics*

The crystalline drugs or antibiotics were ground with a mortar and pestle and thoroughly mixed with the pulverized dog biscuit. The cockroaches did not seem to object to the taste and consumed a normal amount of food. The levels of the doses were selected so as to be of the order of magnitude of the human daily dose with the difference that these doses were consumed throughout the insect's life. The levels were calculated on the basis of the known food consumption during the 300 ± days of a cockroach's life. A second level of doses was then mixed by arbitrarily adding 2 or 5 times the first amounts. The entire series of supplemented diets was as follows:

1) dog biscuit control
2) dog biscuit + 0.1% aureomycin
3) dog biscuit + 0.2% chloromycetin
4) dog biscuit + 1.0% sodium sulfathiazole
5) dog biscuit + 1.0% succinyl sulfathiazole
6) dog biscuit + 0.5% aureomycin
7) dog biscuit + 1.0% chloromycetin
8) dog biscuit + 2.0% sodium sulfathiazole
9) dog biscuit + 2.0% succinyl sulfathiazole

A second series made of a semi-synthetic diet with the same amounts of antibiotics was also fed, but the diet itself affected the bacteroids and those results will be reported in a separate paper (Brooks and Richards, 1955c).

The first time the diets were fed, divided litters were put on the diets as explained under Materials and Methods. Subsequently the experiment was repeated on a larger scale, one entire brood (usually 36 nymphs) being put on each diet and kept at a constant temperature of 27.5° C. The results of both experiments were comparable. When the roaches began to mature (at about 50–60 days), males and females from each diet were sectioned and stained with hematoxylin and erythrosin, and excised fat bodies from additional specimens from each diet were stained *in toto* with neotetrazolium chloride. The fat bodies of all roaches on the *low levels* of antibiotics appeared to have the normal number of mycetocytes, and the bacteroids were viable as judged by their ability to reduce tetrazolium. The fat bodies of the roaches on the *high levels* of aureomycin and sodium sulfathiazole had a reduced number of mycetocytes and the bacteroids in some of the remaining mycetocytes were no longer able to reduce tetrazolium. In fact, at the age of ninety days, other insects on these latter two diets were examined and no bacteroids could be found, either viable or otherwise. The ovaries of some of the females showed signs of deterioration. But all of the roaches on these diets were dead in six months (normal life span is about six months for males and one year for females). The fat bodies of the roaches on chloromycetin and succinyl sulfathiazole at either level were normal.

Aureomycin at both levels, sodium sulfathiazole at both levels, and succinyl sulfathiazole at the high level delayed the maturation of the cockroaches. These substances also caused a delay in the appearance of the first egg capsules, but more significantly, each female usually formed and aborted several egg capsules before one succeeded in hatching. The abortive egg capsules shriveled and dropped off after a few days, while German roaches normally carry their egg capsules for the period of incubation which is 21 to 28 days. No progeny were produced at all on the high level sodium sulfathiazole.

The growth of the roaches on the 2.0% sodium sulfathiazole was so slow that the first adults did not appear until two or two-and-a-half times the period required by the controls.

Although mortality on all of the high levels was considerable—25–50% after three or four months—the life spans on the low levels were not drastically shortened. The ages at death of one group (both sexes) on the dog biscuit control were between 224 and 396 days, while one group on 0.1% aureomycin lived for 187 to 348 days. The other experimentals lived to ages intermediate between those of the controls and the aureomycin-fed group. All of the early deaths on aureomycin were those of males. While the life span of normal males is between five and seven months, aureomycin-feeding shortened the life of males by as much as two months.

In short, administering antibiotics did not eliminate the bacteroids from the fat body of the cockroaches unless the dose was so high that it was accompanied by excessive mortality. However, the effect on the progeny of the treated roaches was quite another matter.

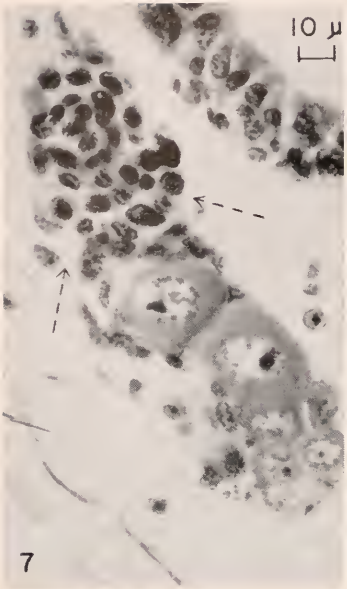
It was immediately obvious that there was something wrong with the offspring of the aureomycin-reared parents. These nymphs were slightly smaller than normal, they were light gray in color instead of dark blackish-brown, and the embryonic cuticle, which is shed at the time of hatching, was not completely cast off but remained crumpled and attached to the anal cerci. The nymphs were weak and feeble. Some of them died immediately, and others lay on their backs for several days waving their antennae; but most of them were strong enough to withstand carbon dioxide anesthesia and careful handling. They were removed from the parental cages and fed pulverized dog biscuit. Most interesting was the nearly complete inability of these nymphs to grow on the stock diet. Control German nymphs on dog biscuit at room temperature molt every ten days, reaching the adult molt at approximately the age of sixty days. The individuals of this generation following aureomycin diet had not molted once by the end of thirty days, although exclusive of the deaths immediately following hatching, mortality was not much higher than among normal roaches and the nymphs ate and were lively.

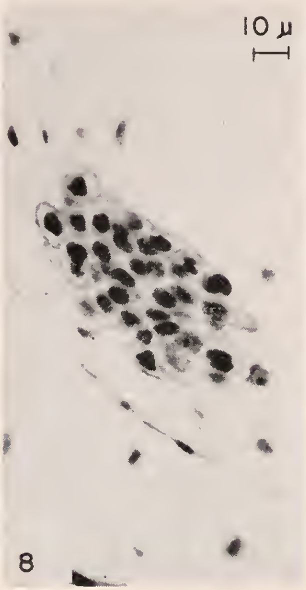
Stained sections of representative samples of the nymphs revealed that they completely lacked bacteroids. In other words, we finally had aposymbiotic cockroaches! Usually no more than 24 nymphs hatched in each brood, whereas between 36 to 44 nymphs usually hatch from normal egg capsules. From every brood, 6 nymphs were taken at random and fixed for histological study while the remainder were used for growth studies. Complete sets of serial sections of the entire insects were carefully examined. No bacteroids were found. The fat body looked exactly like that of normal insects in areas in between mycetocytes except that in the aposymbiotic nymphs there were regions of anomalous tissue which later proved to be the "empty mycetocytes."

The appearance of the aposymbiotic fat body is shown in the photomicrograph, Figure 4, which may be compared with Figure 1, a photomicrograph of a cross-section of a normal nymph containing bacteroid-filled mycetocytes. Figure 7 shows one of the clusters of empty mycetocytes near an ovary of a young nymph. Figure 8 shows another cluster of empty mycetocytes in a more posterior abdominal segment.

Chloromycetin-produced offspring were normal in both histology and growth. A different type of response was elicited, however, by feeding either of the sulfa drugs. Offspring of parents on these diets were of three kinds: 1) normal in both histology and growth; 2) aposymbiotic; or 3) delayed in embryonic development. All three kinds of nymphs occurred in any one egg capsule. There were some bacteroids in the delayed nymphs, but the bacteroids were not enough to fill all of the mycetocytes. Consequently there were a few normally-filled mycetocytes, a few completely empty ones, and numerous partially-filled ones (Fig. 9). As a result of this inadequate complement of bacteroids, such delayed nymphs grew very poorly at first, but after a variable length of time (about a month), they began to grow and they matured at the age of approximately ninety days. If such delayed specimens were examined histologically after normal rate of growth had started, they looked normal. The mycetocytes apparently had become filled and distributed throughout the fat body.

The question logically arises: By what mechanism do the antibiotics break the chain in the hereditary transmission of the symbiotes to the next generation?





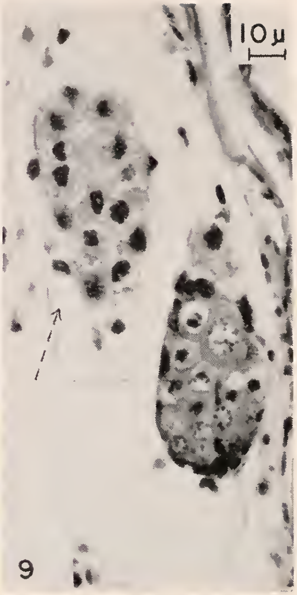


FIGURE 7. Part of a cross-section of a similar nymph as in Figure 4. A cluster of empty mycetocytes (indicated by arrows) is near the ovary. The nuclei of the mycetocytes are prominent but the cytoplasm is almost negligible in amount. The oöcytes are in the lower right side of the photograph. Prepared as in Figure 1.

FIGURE 8. Part of a cross-section of an aposymbiotic nymph showing a cluster of empty mycetocytes in the abdomen posterior to the ovaries. Prepared as in Figure 1.

FIGURE 9. Part of a cross-section through a 12-day old semi-aposymbiotic nymph, *i.e.,* one whose mycetocytes have an inadequate complement of bacteroids and are therefore delayed in development. The mycetocytes have not yet separated as they should normally have done during embryonic development. The ovary is on the right. Prepared as in Figure 1.

The break could be visualized by examining entire ovaries excised from young adult females reared on the various diets. The ovaries were stained with neotetrazolium chloride in such a manner that only the bacteroids were colored, all other tissue remaining colorless (Brooks and Richards, unpublished data).

Before elaborating on these results, it must be emphasized that the normal method of transmission is via the eggs to the embryo, and that the penetration of the ovaries by the bacteroids occurs at an early age. Figure 5 is a photomicrograph of a section of an ovary of a 3-week old nymph, in which some bacteroids have already left the mycetocytes and are now inside of the tunica propria. Such a preparation as this can be obtained equally well from roaches feeding on aureomycin. The antibiotic does not prevent the original infection of the ovaries.

But the tetrazolium-stained ovaries of mature insects show that the effect of the aureomycin takes place in the oldest egg of each series in the ovarioles. In an ovary of a normal female, the rose-violet color of the stained eggs is restricted to the periphery and is due to the reduced dye in the layer of bacteroids. The youngest oöcytes are stained intensely. The mature egg in each ovariole is lighter in color because the rapid enlargement of the ripening egg stretches thin the layer of bacteroids. In an ovary of an aureomycin-reared female, some of the youngest ovules are colored, but the color lessens in the progressively older eggs until the oldest ones are completely white.

All of the examined females that had been reared on dog biscuit had at least *some* bacteroids in *some* of the immature ovules regardless of the antibiotic. On all diets that resulted in nymphs with bacteroids there were also stained bacteroids in the ripe eggs. Those cases which produced normal nymphs had ripe eggs deeply stained, while those cases which produced delayed mycetocytes had only a few bacteroids, probably less than 1% of the normal number, in the ripe eggs; so that grossly the eggs looked light pink or white instead of rose-violet. Those cases which produced nymphs without any bacteroids had very few bacteroids in the immature ovules and none in the ripe eggs. In most of these insects there were viable bacteroids in the fat-body mycetocytes. This leaves the oldest egg, which is rapidly growing, as the first site of complete destruction of the bacteroids.

The aureomycin effect is obtained only by feeding the antibiotic incessantly. This was determined by feeding one group (A) of cockroaches aureomycin (0.1%) until they matured, at which time they were transferred to control diet. Conversely, another group (B) was fed control diet until they matured, when they were transferred to aureomycin. In this way, the females of group A were eating normal diet while their eggs matured. The first nymphs of this group had either no bacteroids or at most only three or four per mycetocyte. During the first nine weeks following removal to normal diet, the successive hatches of nymphs had more and more bacteroids until finally the mycetocytes were normal. The first offspring of group B, which were eating aureomycin while the eggs matured, showed unmistakable signs of malformation of the mycetocytes; and the effect became more pronounced until by thirteen weeks the nymphs were completely aposymbiotic. Thus aureomycin began to affect the eggs within one week but the transmission was not completely blocked until after about three months.

The growth of the nymphs resulting from both groups A and B was directly proportional to the amount of normal mycetocytes in their fat bodies. That is, nymphs that grew well were found to have numerous mycetocytes; those which remained stationary lacked mycetocytes; while an intermediate series, which grew poorly, eventually possessed a few gigantic mycetocytes. The giant mycetocytes seemed to be the result of unchecked growth of the few mycetocytes which had each received only three or four bacteroids from the egg (Brooks and Richards, 1955a). The various subnormal mycetocytes in these nymphs also became attached to the ovaries.

As mentioned earlier, aureomycin shortens the life span of males; but it also, in some unknown way, affects the ability of the males to fertilize the eggs. When both sexes were reared on the antibiotic, there was a high percentage of inviable eggs. If aureomycin-reared females were mated to normal males, most of the eggs hatched. In fact, the analysis was carried one step further by mating normal females to aureomycin-reared males, which resulted in almost as many inviable eggs as from treatment of both sexes.

Seemingly the effect on the males is not one of behavior, as they were observed copulating. It is more likely a direct effect of the symbiotes on the sperm. In normal males, mycetocytes are found close to or attached to the testes (Fig. 6), although bacteroids have never been seen in the testes and there is no evidence for transmission of bacteroids via the sperm. In aposymbiotic males, the empty mycetocytes also migrate to the testes as they do to the ovaries. The lack of bacteroids impairs the reproductive capacity of the aposymbiotic generation males to about the same extent that aureomycin does in the first generation. More will be said of this below.

3. Growth of aposymbiotic nymphs on crude natural diets

Reference has frequently been made to the fact that aposymbiotic nymphs, which themselves have not been fed drugs, are incapable of normal growth on the same crude diet, consisting of well-balanced natural foods, which supports growth of symbiotic cockroaches. An effort was made to replace the function of the bacteroids by feeding them to the nymphs. To this end, pieces of fat body (with bacteroids) freshly excised from normal nymphs were mixed with ground dog biscuit, a little sugar, and water to make a paste. This was renewed twice weekly. Similarly dried brewers yeast and alcohol-insoluble liver fraction (Nutritional Biochemicals Company) were also made into pastes and fed. The nymphs eating the yeast grew slowly and eventually all matured between the ages of 140–154 days, compared to 60 days for normal nymphs. Those eating the liver fraction grew even more slowly, the first one maturing only at the age of 189 days. The fat body did not enhance growth at all. The nymphs on this diet, as well as on the unsupplemented diet, were still immature at 266 days and weighed only between 4 and 7 milligrams. (The adult weights of German roaches are fifty milligrams for males and a hundred milligrams for females. There is no sex-correlated difference until the last instar.)

The cuticle of the slowly growing aposymbiotic nymphs was tan rather than the normal black and brown.

Although eating bacteroids did not improve growth, eating the excreta (accidentally or otherwise) of normal cockroaches did have a slight beneficial effect. Nymphs put in cages with normal nymphs, with normal adults, or even in cages without other insects but which had been soiled by them, all grew significantly better than nymphs isolated in clean cages. Moreover, the ones in the empty but soiled cages grew best of all. One individual has been kept as a curiosity with adults and is now over a year old and about half grown. Evidently the accessory growth factor is present only in the excreta and the improved growth in the presence of other insects is not a trophallaxis.

A few of the nymphs that were fed fat body or excreta were sectioned and stained. It was found that they had not become reinfected with bacteroids. (For the results of implanting tissue, see Brooks and Richards, 1955b).

Since feeding fat body was ineffective, mixing the diets as pastes was unnecessary and in the next trial the diets were fed as dry powders. The supplements were dried brewers yeast, dried alcohol-insoluble liver fraction, dried egg yolk (spray process, Fletcher-Eichman Company), and uncooked wheat germ (breakfast cereal, ground with mortar and pestle). A fifth diet was made of equal parts of each of the other four so that the final percentage of any one of the supplements was one-fourth as much as when it was the only supplement. Figure 10 shows

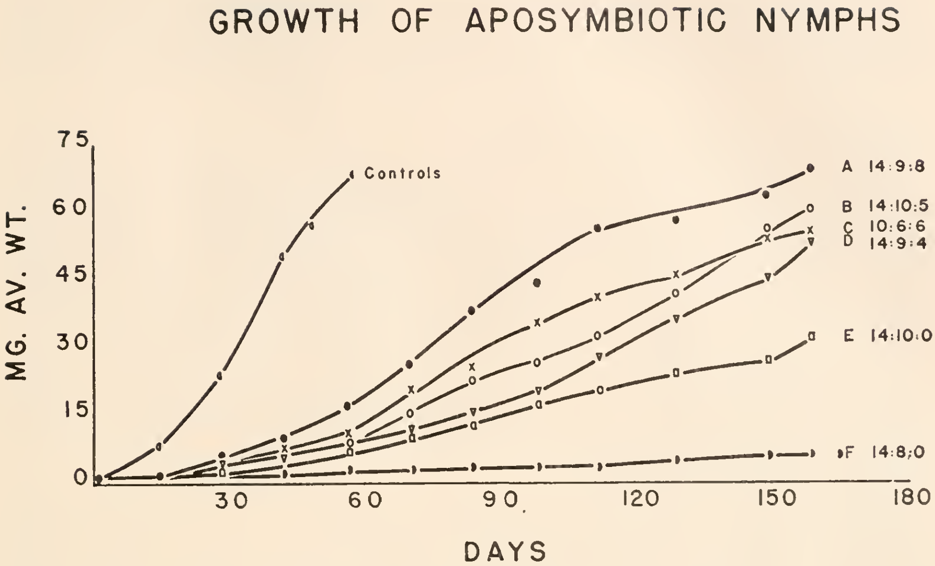


FIGURE 10. Growth of normal and aposymbiotic German cockroach nymphs. The insects were reared at approximately 25° C. The controls were normal nymphs fed unsupplemented dog biscut. They had all reached maturity by the age of 57 days. The aposymbiotic nymphs were fed dog biscuit with supplements as follows (except F): A, 50% dried brewers yeast; B, 30% fresh wheat germ; C, equal parts A, B, D and E: D, 15% dried egg yolk; E, 10% dried alcohol-insoluble liver fraction; F, unsupplemented. The numerals after the key letters indicate the number of nymphs at the start of the experiment : the number alive when the experiment was terminated : the number which had matured.

the growth curves obtained. The numbers following each label represent the number of insects at start : number of insects at finish : number of insects maturing to adults. It can be seen that again a diet of 50% brewers yeast enabled the nymphs to mature in about two or three times the period required by normal nymphs. This seems to be an inordinately high amount of yeast, but in several preliminary trials, percentages of 25, 12.5, 10, and 5 were less effective in that order.

In order to dispel any doubts that it is the lack of bacteroids instead of a carrying-over of toxic effects of the antibiotics which prevents growth, we kept individual records on the performance of one litter from sulfa-fed parents. Six of the nymphs were killed immediately after hatching and they established the expected pattern of part symbiotic, part aposymbiotic nymphs. Twenty remaining nymphs were equally and randomly distributed to diets consisting of dog biscuit control, and yeast, liver, and fat-body supplements. It turned out that there was at least one insect with bacteroids on each diet, and each of these insects grew well and matured. The roaches without bacteroids grew poorly and failed to mature on the control and fat body-supplemented diets. The roaches without bacteroids grew slowly and eventually matured on the yeast- and liver-supplemented diets. These were not guesses: the insects were weighed individually and each one finally sectioned and stained. Since the nymphs were litter-mates, any toxic effect of the drug fed to the mother should be expected to have affected them all alike. On the contrary, the lack of growth was directly correlated with the lack of bacteroids.

4. *Reproduction of aposymbiotic roaches*

Crosses were made between the three possible combinations of normal and aposymbiotic roaches after they had matured on the high yeast diet. The absence of the bacteroids did not result in early dissolution of the ovaries nor in complete suppression of the capacity for reproduction. However, there was a definite inhibition of reproduction, and this was true of males as well as of females. Continued feeding of yeast to the adults did not improve reproduction over that on an unsupplemented diet.

The method of testing these crosses was as follows: each aposymbiotic female was paired with a normal male; and each aposymbiotic male was mated to three recently emerged virgin females. A record was kept of the number of egg capsules produced in each cage.

Egg capsules produced by normal cockroaches almost invariably hatch if they are fertilized and if they are not damaged. However, 7 aposymbiotic females mated to normal males produced a total of 11 abortive egg capsules before nymphs hatched from 2 capsules. These and all subsequent offspring were without bacteroids. The 18 normal females mated to 6 aposymbiotic males produced 10 abortive egg capsules before nymphs hatched from 6 capsules. At the age of 20 days these nymphs molted to second instar, which is a normal rate of growth. When sectioned, they were seen to have normal mycetocytes.

Several litters hatching in the cages in which both parents were aposymbiotic constituted the second generation of cockroaches without bacteroids. Their histology and behavior were the same as those of the first generation. These have now been continued to the third aposymbiotic generation with the same properties being maintained.

DISCUSSION

The endosymbiotic relationship of cockroaches and bacteroids is certainly of great antiquity. The recent discovery of a similar symbiosis in *Mastotermes*, a Carboniferous connecting link between the Isoptera and the Blattariae, places the relationship in the roaches as at least 300 million years old (Buchner, 1953; Koch, 1938).

There are several theories prevalent as to how endosymbiosis arose. Koch (1949) found that the mycetocytes form in the embryo of the German cockroach in anticipation of the reception of the bacteroids. The cells lie at the edges of the segmented fat body adjacent to the mid-gut and after their infection they sink deeply into the fat body. That the mycetocytes persist as specialized cells even in the absence of the bacteroids was established in the experiments reported in this paper. Possibly the mycetocytes originated phylogenetically as specialized cells within the intestinal epithelium, where they become infected with bacteroids contaminating the food. Their evolution would then have been toward removal from the epithelium and complete submersion in the fat body. This position would have necessitated the intervention of ovarian infection to insure transmission.

The restriction of the bacteroids to the mycetocytes may be the result of host immunization, as Glaser (1920) suggested, but the end result is the protection of the bacteroids. The relative imperviousness of the fat body to many influences such as extremes of osmotic pressure, temperature, food, and antibiotics undoubtedly protects the bacteroids within the mycetocytes. The bacteroids are peculiarly susceptible to destruction when they leave the mycetocytes. While in the developing ovum they can be killed by heat or antibiotics, and also in the developing ovum they either perish or fail to reproduce themselves if the host is consuming an incomplete diet (Brooks and Richards, 1955c). Furthermore, the bacteroids failed to establish themselves when they were injected as a suspension into the hemocoel (Brooks and Richards, 1955b).

Cockroaches deprived of bacteroids by breaking the chain of hereditary transmission by any of the methods mentioned above cannot live normally on a crude natural diet that is adequate for symbiotic roaches. There is a high percentage of mortality in the newly-hatched nymphs and growth is extremely slow in the survivors. While it is true that cockroaches are omnivorous, their food supply is meager at best and frequently unbalanced. If the diet of the aposymbiotic nymphs is fortified with highly nutritious foods, growth proceeds but at a slower rate than normal. Reproduction of adult aposymbiotic roaches, both males and females, is also deleteriously affected, most of the egg capsules, particularly the first several, being non-viable. The total result of depriving the insects of their symbiotes is thus one of *delay* in both growth and reproduction.

Since the bacteroids can be partially compensated for by a vitamin-rich diet, perhaps the function of the bacteroids is the production of a vitamin(s). But since the amount of vitamin-containing food that is required is out of all proportion to known vitamin requirements, it seems that the factor(s) needed is either 1) unknown and present in low concentration, or 2) not used *per se* but serves as a precursor of a second factor(s), such as a co-enzyme, the synthesis of which is aided by the bacteroids. The bacteroids themselves do not constitute a store of the required substance, because eating bacteroid-containing fat body did not result in re-infection of the aposymbiotic nymphs and did not permit normal growth.

Heat, aureomycin, and sulfa drugs were not equally effective in preventing transmission of the bacteroids to the next generation of roaches. The sulfa drugs were unreliable as they produced variable results. Aureomycin was completely effective and the results could always be duplicated. Heat treatments were not only less effective in preventing transmission, but the high temperature itself was fatal to a majority of the roaches, special equipment was needed for maintaining constant temperature and humidity, and there were difficulties in keeping drinking water available for the insects. In spite of these objections against using heat and sulfa, the results were worth the effort because they gave independent verification of the results from aureomycin. Regardless of which method was used, aposymbiotic offspring had the following characteristics in common: lack of growth on normal diet, slow growth with brewers yeast added to the diet, light colored cuticle, and poor reproduction.

First generation roaches suffering destruction of most of their bacteroids by either heat or a high level of sodium sulfathiazole stopped growing, but when yeast was added they resumed growth.

We have no suggestions to offer as to the processes by which the high temperature (37° C.) destroyed the bacteroids, but it seems appropriate to mention at this time that there are numerous reports in the literature on the inability to culture bacteroids at the standard incubator temperature of 37° C.

The specific effect of low level aureomycin on the bacteroids in the eggs as against those in the fat body is provocative. There may be a more rapid rate of metabolism in the enlarging eggs so that the antibiotic turn-over is stepped up, thus effectively increasing the dose acting on the bacteroids. This turn-over may be thought of as mitigated by either the egg protoplasm or by the bacteroids. On the other hand, instead of a quantitative difference between eggs and fat body, there might be a qualitative difference between the metabolism of the two tissues. This probably could occur if the chemical structure of the antibiotic resembles that of certain precursors needed for building egg protoplasm. And finally, there is the possibility that the bacteroids in the ripening eggs are simply prevented from reproducing themselves rather than killed outright.

There is no obvious reason why the various antibiotics should have acted as differently as they did. According to Merck's Index (1952), aureomycin is active against certain Gram-negative and Gram-positive bacteria, rickettsiae, protozoa, and viruses. Chloromycetin is active against all the preceding organisms except the protozoa, while sodium sulfathiazole is active against many bacteria. The effect of succinyl sulfathiazole is surprising inasmuch as this drug is commonly thought not to be absorbed and therefore effective only against enteric bacteria.

Theoretically, one may object to admitting that the evidence presented in this paper proves that the bacteroids are necessary for normal growth. The objection states that the bacteroids may be accumulated products of growth, and since the nymphs cannot grow as a result of the drugs given their parents, the bacteroids are not accumulated. However, stained sections of aposymbiotic roaches which *did* grow to maturity on yeast-diet contained no bacteroids. If the bacteroids are products of growth, they should have been accumulated in these insects.

The whole problem of the relationship of the bacteroids to the testes needs to be thoroughly investigated. The anatomical association of the mycetocytes with the testes and the impaired fecundity of both aureomycin-fed and aposymbiotic males all indicate that the bacteroids are of more significance to the male than has hitherto been suspected.

The function of the bacteroids as related to reproduction seems to be the supplying of a factor which, in the absence of the bacteroids, is not necessarily absent but available only in small amounts. The presence or absence of the bacteroids does not have an all-or-none effect on either reproduction or growth.

We wish to thank Dr. Jerre L. Noland for numerous valuable suggestions, and especially for pointing out the advantages of the German cockroach for the particular experiments needed.

SUMMARY

1. The trans-ovarial inheritance of fat-body intracellular symbiotes in the German cockroach was prevented by subjecting the parent insects to high temperature or by feeding the parents aureomycin or sulfathiazole.

2. The most certain and simple method of obtaining aposymbiotic nymphs is by feeding the parents ground dog biscuit plus 0.1% aureomycin *all* of their lives.

3. Aposymbiotic nymphs are practically incapable of growth on a natural diet which is adequate for symbiotic nymphs.

4. The addition of large amounts of dried brewers yeast to the diet enabled the aposymbiotic nymphs to grow to maturity in two to three times the period required by normal nymphs.

5. Adult aposymbiotic cockroaches suffered impaired reproductive ability. The males were affected as well as the females.

6. The second and third generations of aposymbiotic roaches are similar to the first in both histology and behavior.

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STUDIES ON THE ACROSOME. IV. THE ACROSOME REACTION IN SOME BIVALVE SPERMATOZOA[1]

JEAN C. DAN AND SEIJI K. WADA[2]

Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan

An earlier study (Dan, 1952) has demonstrated that the spermatozoa of several sea urchin species respond to certain stimuli by a characteristic reaction of the acrosome, in which the membrane covering its anteriormost part appears to break down, exposing the underlying substance. Part of this exposed material disperses almost immediately, leaving a short filamentous structure which remains essentially unchanged thereafter. This reaction is induced by egg-water of the same species, by alkaline sea water (pH 9.2) and by contact of the spermatozoa with such surfaces as glass and collodion.

In starfish spermatozoa, an acrosome reaction of a rather different sort takes place (Dan, 1954). The nearly spherical heads of these spermatozoa are flattened anteriorly, and the acrosome is imbedded in the nuclear region. If the spermatozoa are suspended in a 0.5% solution of crystallized egg albumin in sea water (as adjuvant), and then mixed with egg-water of the same species, a very long (ca. 25 μ) straight filament is extruded from the center of the acrosome surface and simultaneously certain changes take place in the interrelations of the head, middle piece and tail. The diameter of this filament is somewhat less than that of the axial filament of the tail, and it possesses considerable rigidity. Like the much shorter filaments of sea urchin sperm, these structures are relatively durable in sea water.

It has been found that the spermatozoa of several representative bivalve species undergo a similar reaction. These animals were chosen as objects for the study of the acrosome reaction because in many species of this class fertilization takes place externally and can be accomplished experimentally. Some of the species available in the vicinity of Misaki have been observed in their respective breeding seasons; the sampling includes representatives from three of the five orders, and eight families.

These animals generally have spermatozoa in which it is easy to identify the acrosome with the high power of the phase contrast microscope, and to differentiate it from the other components of the sperm head. In some of the species examined in this study, the stimulus of contact, alone or in the presence of egg-water, causes the disappearance of the original structure, and there appears in its place a filament of about the same diameter as that of the starfish filament. In the other species, the same sort of reaction was observed to have taken place in (supernumerary) spermatozoa in the vicinity of the egg surface at fertilization.

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[2] Present address: Faculty of Fisheries, Kagoshima University, Kagoshima, Japan.

This complete disappearance of the acrosome in *Mytilus edulis* was reported by Kupelwieser in 1909 and by Meves in 1915. Both workers found that the sperm head just inside the cytoplasm of eggs immediately after fertilization lacked any sign of the "perforatorium." Meves comments (p. 53): "Merkwürdig ist, dass das Perforatorium nicht nur bei den völlig aufgenommenen Köpfen, sondern auch schon bei den noch im Eintritt begriffenen spurlos geschwunden ist; sein Substanz muss also sehr rasch auflösen."

This paper will deal with the morphological aspects of the acrosome reaction in twelve species of bivalve molluscs.

Materials and Methods

Observations were made on the spermatozoa of the following Pelecypoda:

Mytilidae
    Mytilus edulis Linné
    Lithophaga curta (Lischke)
Spondylidae
    Spondylus cruentus Lischke
Ostreidae
    Crassostrea (= *Ostrea*) *echinata* (Quoy et Gaimard)
    Crassostrea (= *Ostrea*) *nippona* (Seki)
    Crassostrea (= *Ostrea*) *gigas* (Thunberg)
Trapeziidae
    Trapezium sublaevigatum (Lamarck)
Chamidae
    Chama retroversa Lischke
Petricolidae
    Petricola japonica Dunker
Mactridae
    Mactra veneriformis Reeve
    Mactra sulcataria Reeve
Pholadidae
    Zirfaea subconstricta (Yokoyama)

In this survey, attention was directed to those species in which artificial fertilization was known to be possible, and in every case, the utmost effort was made to induce spawning, because it was early found that the reacting capacity of spawned spermatozoa is generally much greater than that of sperm taken from excised testes. Methods for inducing spawning of bivalve molluscs have been reported by various workers; they include exposure of the animals to sperm- or egg-charged sea water (Galtsoff, 1938, 1940; Wada, 1954), electrical stimulation (Iwata, 1949), raising of water temperature (Wada, 1936; Galtsoff, 1938, 1940). Variations and combinations of these methods were often found effective; in some of the species reactive spermatozoa were obtained by simple excision of the testes, or by excision following some kind of stimulation.

The animals were used as soon after collection as possible, and were always kept in running water in the laboratory. When increased temperature was used to induce spawning, the incoming sea water was run through a glass coil immersed in a vessel of water heated by an electric heating unit; the temperature of the water surrounding the animals was regulated to 25–28° C. by adjusting the rate of flow.

Sperm- and egg-suspensions for inducing spawning were prepared by adding the gametes from an opened male or female to a suitable volume of sea water. These suspensions were added to the sea water containing the animals, sometimes alternately, and often following an extended period of warming. In some cases animals were induced to spawn by the second or third such stimulus, after having been continuously at the high temperature for several hours.

Electrical stimulation was used particularly in the case of *Mytilus*, following the method of Iwata. The shells of the mollusc are held apart by a wooden wedge about 5 mm. thick, and a small roll of absorbent cotton moistened with sea water is partially inserted into the space between the shells near the narrow end, so that it comes into contact with the tissues. The animal is then supported over a vessel filled wih sea water so that the wide end is immersed in the sea water. A variable resistance is interposed in the ordinary (50 cycle) alternating current and adjusted so that about 20 volts passes through the completed circuit. One Ag-AgCl electrode is dipped into the sea water, while the other is brought into contact with the cotton wad for 15 seconds. The wedge is then removed and the stimulated animals placed in sea water in separate containers until they spawn.

Egg-water for inducing the acrosome reaction was prepared by the method used for echinoderms. Unfertilized eggs are suspended in a small volume of sea water and left standing, usually for 30 minutes or more. The supernatant of this suspension is filtered before use.

As in the case of starfish spermatozoa, the acrosome filaments of bivalve sperm are not satisfactorily preserved in mass suspensions by formalin fixation. With this method, even with very dilute formalin, the material of the filament, when it can be detected at all, is in the form of a rounded mass on the anterior part of the sperm head. It is possible, however, to obtain a rather unsatisfactory fixation with formalin of filaments which are stuck on a glass, collodion or formvar surface. With osmic vapor, the filaments are well preserved, but the sperm heads are distorted by swelling, and often break down entirely on subsequent washing and drying.

The most satisfactory observations were made by placing a small amount of living sperm suspension on a coverglass which was inverted on a slide and observed with the phase contrast immersion objective, using anisol ($C_6H_5 \cdot O \cdot CH_3$—refractive index = 1.515) as the immersion fluid. (Since this substance has a low viscosity, its use instead of cedar oil obviates the necessity for fixing the coverglass in place.) The spermatozoa which have reacted on contact with the coverglass become attached to its under surface, where they are most successfully observed. Unfortunately, at this magnification (ca. 1000 ×) the focal depth is so shallow that it will not include both the filament on the underside of the coverglass and the outline of the sperm head about 1.5 μ below it. For this reason photography was not practicable, and records had to be made in the form of camera lucida drawings or free-hand sketches.

RESULTS

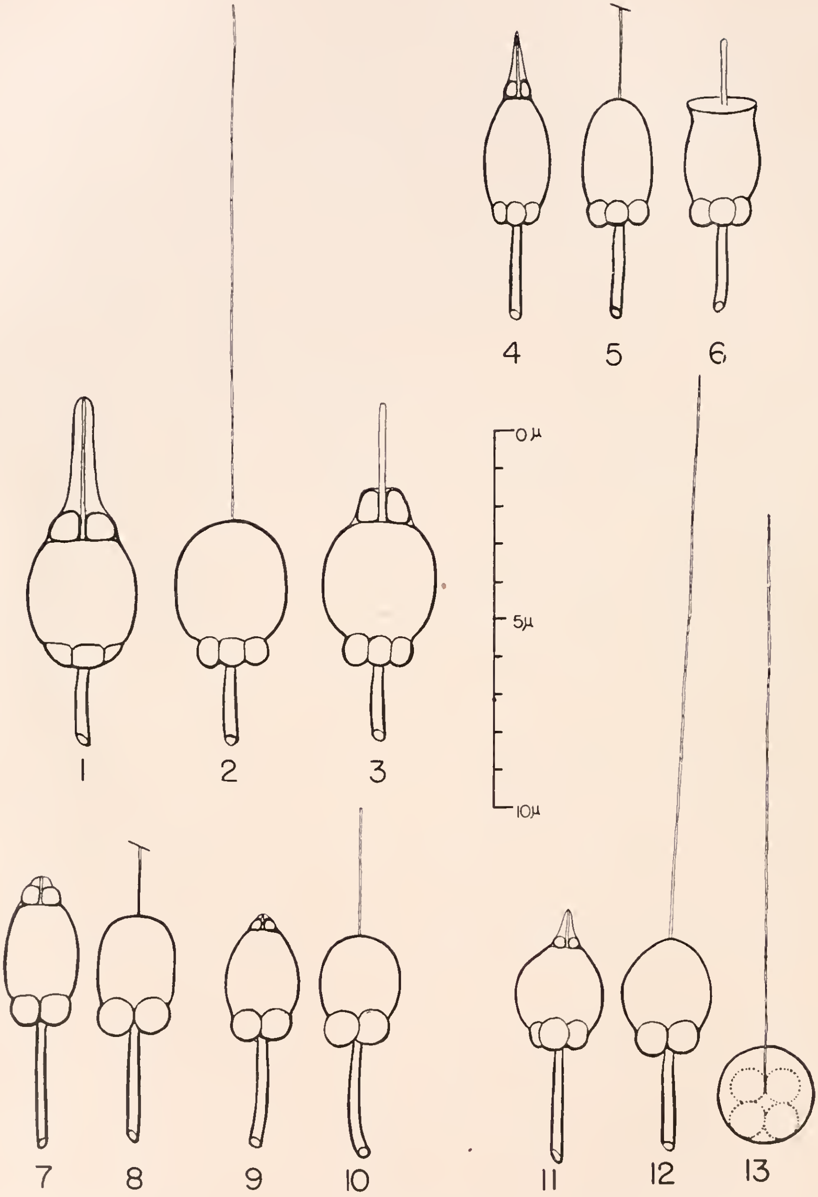
The spermatozoa of all these species resemble each other more or less closely in such fundamental characteristics as the shape of the nuclear part of the head, and the fact that the middle piece takes the form of four or five small spheroidal bodies arranged in a ring around the insertion of the flagellum. The acrosomes are very different in size, but always appear as cones affixed to the anterior part of the nucleus. In some cases the cones are so low that it is barely possible to establish their existence, but in the species with conspicuous acrosomes there is clearly visible an axial differentiation, which might represent either a tubular passage through the center of the acrosomal cone or a fibrillar structure.

When the acrosome is large enough so that its substance can be distinguished from the surrounding membrane, it appears (with phase contrast, dark contrast) to be filled with a rather strongly refringent hyaline material. In the largest acrosomes (e.g., that of *Mytilus edulis*) it is possible to see a clearly differentiated basal part which has considerable structural rigidity, and there is evidence that such a structure is also present in small acrosomes. In 1% OsO_4-sea water, this structure and the spheres making up the middle piece are not blackened after 30 minutes' fixation.

In all the twelve species examined, the reaction of the spermatozoa to egg-water, or to contact with an egg or other surface such as glass or collodion, results in a complete disappearance of the original acrosome. In its place there appears a slender, rigid filament, extending directly forward from the center of the area previously covered by the acrosome. It is difficult to estimate the diameter of these filaments, since it is at the limit of resolution of the optical system when they are observed in the living state with the light microscope, while measurements of electron micrographs involve several serious sources of error, such as possible shrinkage on fixation, and undoubted distortion (flattening and shrinkage) during mounting and desiccation in preparation for observation *in vacuo*. Both in the living state and in electron micrographs the acrosome filament appears to be of approximately the same diameter as the axial filament of the sperm tail.

In the course of these observations it became evident that the reacted (supernumerary) spermatozoa which are seen around an egg after fertilization do not, as was first assumed, represent individuals which have responded to the stimulus of substances from the egg at a little distance from it and have been prevented, by some monospermy-insuring mechanism, from penetrating its surface. There is evidence that the spermatozoa in these molluscan species react only after they have established contact with the vitelline membrane, the acrosome filaments of all the reacting spermatozoa first extending into the egg cytoplasm, and later all but the fertilizing spermatozoan being expelled. Since this process apparently involves a shortening of the acrosome filament, the true filament length of these supernumerary sperm may be considerably greater than that observable by the methods hitherto used. This question will be discussed in a later paper.

It is a common characteristic of all the observed species that reacted spermatozoa show a reduction in activity, together with a "loosening" of the head structure, similar to that which occurs in the starfish sperm. This is most clearly observed in a changed appearance of the spherules making up the middle piece. In the spermatozoan before reaction, these are usually much compressed so that it is often



Figures 1–29. Sketches of some bivalve spermatozoa, showing morphological changes resulting from the acrosome reaction.

Figure 1. *Mytilus edulis* spermatozoan in sea water.
Figure 2. *Mytilus* sperm after reaction induced by egg-water.
Figure 3. *Mytilus,* partial reaction.
Figure 4. *Petricola japonica* spermatozoan before reaction.

difficult to determine their number, and their outline is often more or less continuous with the curve of the sperm head. After the reaction, on the other hand, they are more conspicuous as separate spheres, as though a tight enveloping membrane had been relaxed (*cf.* Figs. 1 and 2, 4 and 5, etc.). There is also a tendency, in elongated spermatozoa, for the anterior part of the nucleus, after the breakdown of the acrosome, to lose the truncated cone shape and become more nearly spherical (Figs. 7 and 8, 17 and 18).

In the following section, the methods used for obtaining gametes and the characteristics of the spermatozoa and their acrosome reaction are reported for each species. The approximate breeding season at Misaki is indicated in parenthesis.

Mytilus edulis (Autumn and winter)

Spawning was induced by electrical stimulation. During most of the breeding season these animals begin to shed eggs or sperm about 40 minutes after being stimulated at 13–15° C., and within 30 minutes at 18–20° C. Shedding males were usually removed to dry Syracuse watch glasses, so that the sperm could be obtained with a minimum of dilution.

The large *Mytilus* spermatozoan has a strikingly prominent, pointed acrosome (Figs. 1, 30), which is 3.5 to 4.5 μ in length, somewhat more than the combined length of the nucleus and middle piece. Through the center of this extended cone there is a clearly visible axial structure, and at its base, a differentiated region which appears in living spermatozoa as a thick ring forming the base of the acrosome.

These spermatozoa undergo the acrosome reaction in a relatively high percentage if spawned spermatozoa are mixed with egg-water. In this reaction (Figs. 2, 31), the whole acrosome breaks down, and in its place there is extruded a very slender filament about three times the length of the original acrosome. When the fertilization process is observed with phase contrast high power, such a filament can be seen, extending between the heads of supernumerary spermatozoa and the egg surface. The fertilizing spermatozoan is always closely against the vitelline membrane by the time it can be brought into focus.

It has so far not been possible to observe the acrosome in the actual process of breaking down under the stimulus of egg-water, because of the intense activity of the spermatozoa. However, when they are suspended in 0.05% merthiolate-sea water, the spermatozoa are immobilized at once, and the breakdown of the acrosome follows, but not immediately or simultaneously, so that it is possible to observe the process under these conditions. The acrosome breaks down progressively from

FIGURE 5. Reacted *Petricola* spermatozoan as found in vicinity of egg immediately after fertilization (supernumerary sperm).

FIGURE 6. Partial reaction in *Petricola* sperm.

FIGURE 7. *Mactra sulcataria* spermatozoan.

FIGURE 8. *M. sulcataria* supernumerary spermatozoan.

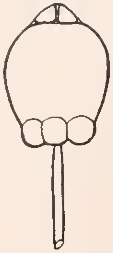
FIGURE 9. *Mactra veneriformis* spermatozoan.

FIGURE 10. *M. veneriformis* spermatozoan after acrosome reaction induced by egg-water.

FIGURE 11. *Spondylus cruentus* spermatozoan.

FIGURE 12. *Spondylus* sperm reacted in egg-water (side view).

FIGURE 13. Reacted *Spondylus* spermatozoan as found affixed to under side of coverglass by adhesive anterior surface.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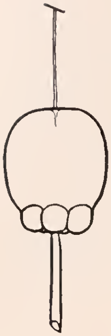
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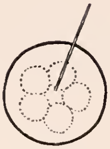
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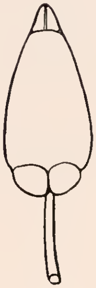
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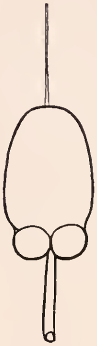
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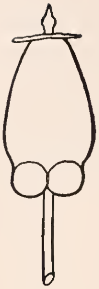
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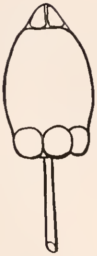
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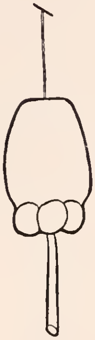
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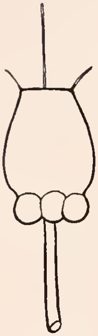
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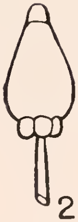
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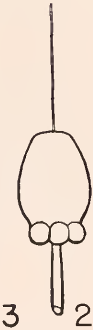
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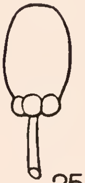
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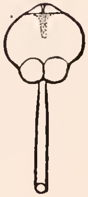
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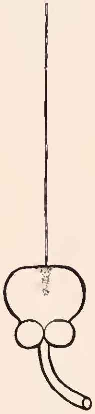
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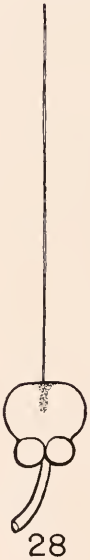
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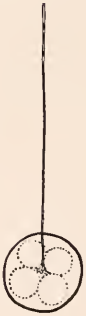
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FIGURE 14. *Zirfaca subconstricta* spermatozoan.
FIGURE 15. Supernumerary *Zirfaca* sperm in side view.
FIGURE 16. Supernumerary *Zirfaea* sperm stuck to coverglass by adhesive anterior surface.
FIGURE 17. *Lithophaga curta* spermatozoan.
FIGURE 18. Reacted *Lithophaga* sperm.
FIGURE 19. Partial reaction of *Lithophaga* spermatozoan.
FIGURE 20. *Chama retroversa* spermatozoan.

the tip, within a fraction of a second, leaving no trace of the original structure except a small remnant at the base. The filament in these cases is shorter and stouter than normal. There is no evidence that the effect of merthiolate is identical with that of substances from the egg—this observation is offered simply as the only presently available information as to the manner in which the process of acrosome breakdown may proceed.

Some further inferences can be tentatively drawn from observation of spermatozoa which have undergone what has been provisionally called "partial reaction" (Fig. 3, *cf.*, Figs. 6, 19). In such spermatozoa, either because of their sub-normal condition or as the result of a sub-optimal stimulus, the reaction has apparently stopped with the breakdown of the distal part of the acrosome, leaving a basal collar-like structure. Such spermatozoa always show a filament which is about the length of the original acrosome.

Lithophaga curta (Late spring)

Males spawned on the third addition of egg-suspension to animals in warm (28° C.) running sea water. The spermatozoa thus discharged were intensely active, and their acrosomes reacted on contact with the coverglass (Figs. 17, 18).

In this species there were many cases of "partial breakdown," in which some material, presumably the covering of the acrosome, remained attached to the anterior part of the head; and a poorly defined pointed rod, corresponding to the axial structure of the intact acrosome, could be seen still in its original position (Fig. 19).

Spondylus cruenta (Summer)

Active spermatozoa were obtained by extirpation of the testes, and these readily underwent the acrosome reaction on addition of egg-water prepared by removing the supernatant from a 5% suspension of eggs after 10 minutes (Figs. 11, 12). Reacted sperm showed a strong tendency to adhere to the coverglass by the anterior surface of the head, with the acrosome filament bent perpendicular to its normal position (Fig. 13). This behavior, which indicates that the breakdown of the acrosome leaves the sperm head surface locally sticky, is also found in the spermatozoa of the oysters and *Zirfaea*. In *Spondylus* the sperm head is slightly asymmetrical bilaterally, and the figure presented by the adhering spermatozoan is correspondingly somewhat off-center.

FIGURE 21. Supernumerary *Chama* spermatozoan.

FIGURE 22. Reacted *Chama* spermatozoan, acrosome covering incompletely broken down.

FIGURE 23. *Trapezium sublaevigatum* spermatozoan.

FIGURE 24. Reacted *Trapezium* sperm.

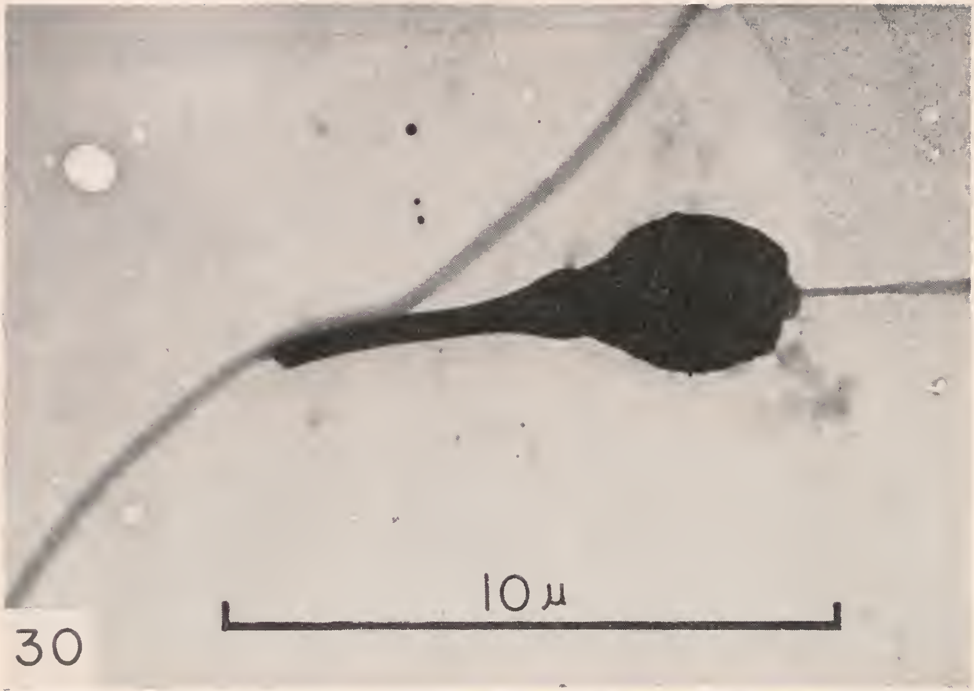
FIGURE 25. *Trapezium* spermatozoan in which acrosome has broken down but no filament is apparent.

FIGURE 26. Spermatozoan of *Crassostrea echinata*, *C. nippona*, *C. gigas*.

FIGURE 27. Spermatozoan of *C. echinata* or *C. gigas*, reacted on contact with glass surface in presence of egg-water (side view).

FIGURE 28. Reacted sperm of *C. nippona*, from side.

FIGURE 29. Anterior view of reacted *C. echinata* spermatozoan attached to coverglass by adhesive surface.



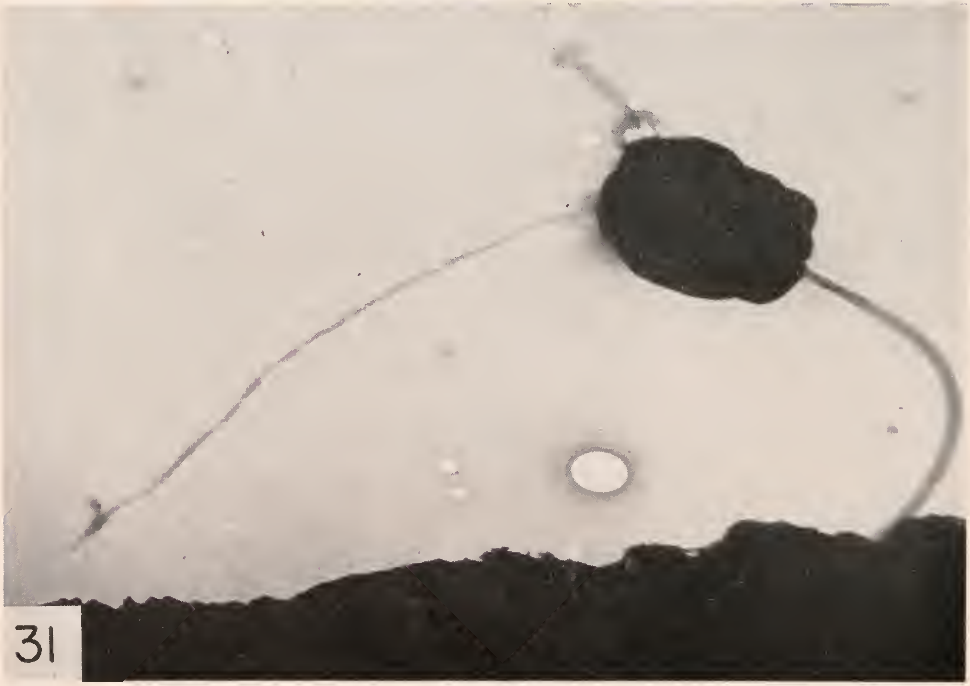


FIGURE 30. Electron micrograph of *Mytilus edulis* spermatozoan fixed with formalin in sea water.

FIGURE 31. Reacted *Mytilus* spermatozoan (formalin fixation).

Fertilization with the gametes obtained in this way was only possible when the pH of the sea water was increased by the addition of NH_4OH to a final concentration of 1.0×10^{-3} N. Filaments were observed connecting the supernumerary sperm with the egg surface.

Crassostrea echinata (Summer)
C. nippona
C. gigas

Spawning was induced by adding egg-suspensions to the tanks containing animals in warm (28° C.) running sea water. Fairly reactive gametes can also be obtained by simply cutting open the animals. It was found that the best method for obtaining a dense suspension of maximally reactive sperm is to cut open a male which has begun to spawn in response to the warming-plus-egg-water stimulus.

The spermatozoa of these three species are indistinguishable both before and after the acrosome reaction, except that the filaments in *C. nippona* are longer than those of the other two species. The intact acrosome is a small, low cone, which is affixed to the anterior part of the nearly spherical head (Figs. 26, 32). Under the center of the acrosome there extends radially inward a gray-appearing (with dark phase-contrast) structure or region not sharply differentiated from the surrounding nuclear material; this is even more clearly visible after the acrosome has reacted, and the acrosome filament seems to extend directly from its center (Fig. 27).

In these species the shape change accompanying the acrosome reaction tends to flatten the anterior surface of the sperm head, which becomes extremely adhesive as the result of the acrosome breakdown (see also *Spondylus*), and sticks to the glass by this surface (Fig. 29).

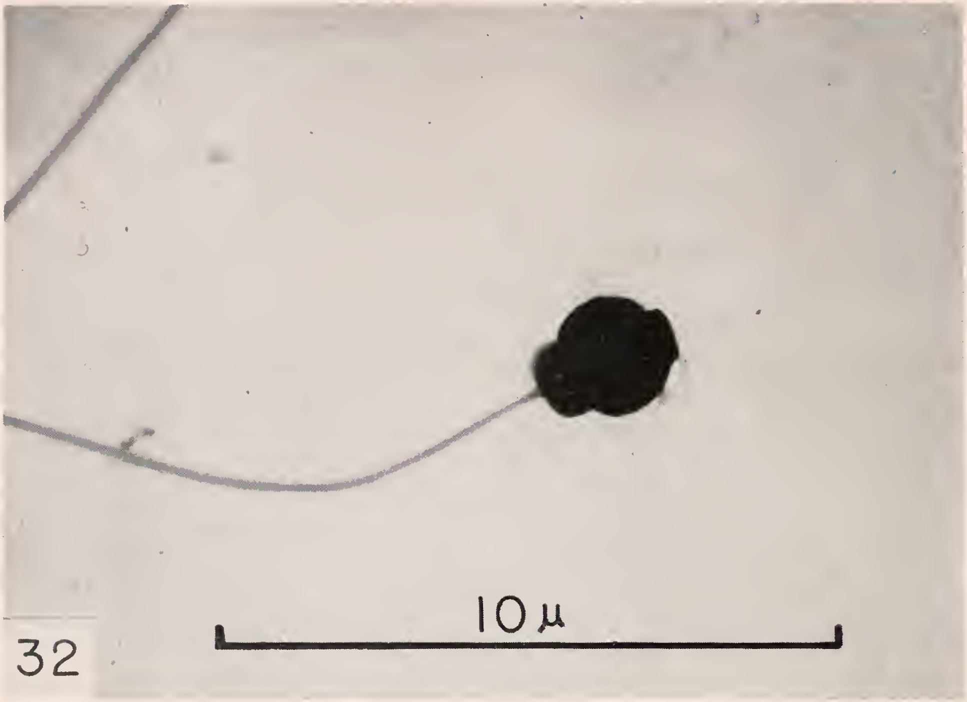
Intermediate stages in the acrosome reaction indicate that the extrusion of the filament occurs simultaneously with the breakdown of the original acrosome surface. It is probable that Figure 33 represents a case in which the normal process was stopped by fixation.

In order to obtain a high percentage of reacted acrosomes, the spermatozoa of these species must be brought into contact with an egg, or with some other surface in the presence of egg-water. It makes no difference whether the eggs are unfertilized, fertilized or even cleaved, and if the suspension is stirred sufficiently, the presence of a few eggs will induce reaction of the acrosomes in most individuals of a relatively dense sperm suspension.

Trapezium sublaevigatum (Late summer)

Gametes were obtained by cutting open the animals. The spermatozoa were intensely active in sea water suspension, but the eggs were very fragile and easily broken in handling.

In the vicinity of the eggs, the spermatozoan acrosomes broke down, and many sperm were observed attached to the vitelline membranes by filaments about 3 μ in length (Figs. 23, 24). In other cases the acrosomes were gone but no filaments were in evidence (Fig. 25). Such spermatozoa were often attached



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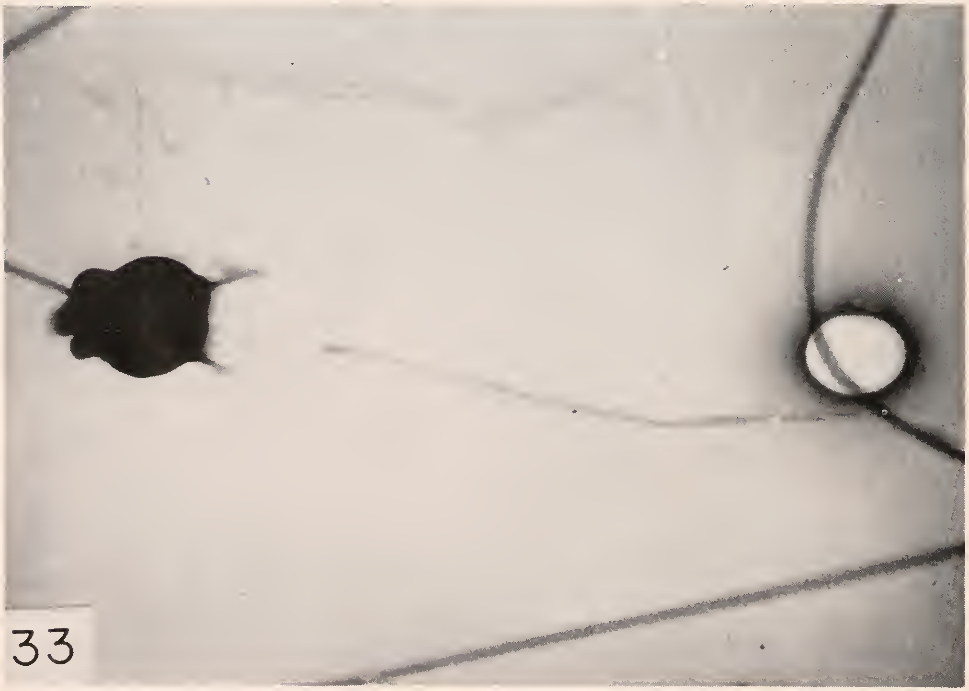

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FIGURE 32. Spermatozoan of *Crassostrea gigas* in sea water. Formalin fixation has somewhat distorted shape of acrosome.

FIGURE 33. *C. gigas* spermatozoan, reacted in response to contact with collodion membrane

obliquely to the coverglass by the anterior part of the head. In these sperm, also, the breakdown of the acrosome apparently leaves the underlying surface sticky, although not as markedly so as in the oysters.

Chama retroversa (Summer)

Sperm and eggs were obtained from opened animals. The spermatozoa showed vigorous activity on suspension in sea water, and fertilization took place readily in normal sea water. In this species also, the fertilizing spermatozoan was already close against the vitelline membrane at the earliest observation, but supernumerary sperm were found in the jelly layer with extruded filaments (Fig. 21). As suggested above, the length of the acrosome filament in such cases is doubtful.

The blunt acrosome in this species (Fig. 20) consists of an axial, differentiated structure surrounded by refringent substance. Not infrequently individuals are found in which the covering membrane of the acrosome has apparently failed to break down completely (Fig. 22).

Petricola japonica (Late spring and early summer)

These animals were induced to spawn by exposure to warm running sea water. Spermatozoa removed from opened animals were quiescent on being suspended in sea water, but became intensely active after a short time. These spermatozoa have a relatively long (ca. 1.7 μ), sharply pointed acrosome (Fig. 4) with a well-defined axial structure. In some cases the acrosome may break down, leaving a filament which is just the length of the original acrosome, and some other material which persists around the base of this filament (Fig. 6). In the complete reaction, as seen in supernumerary spermatozoa (Fig. 5), the acrosome breaks down entirely.

Mactra veneriformis (Late spring and early autumn)

Reactive gametes were secured by opening the animals. The eggs remain fertilizable for several hours if they are left in the body fluid of the clams, but the germinal vesicle breaks down when the eggs are introduced into sea water, and the eggs can then no longer be fertilized. Practically 100% fertilization was obtained with this species in 80% sea water.

The spermatozoa have small acrosomes in which an axial differentiation is just visible (Fig. 9). The heads of completely reacted spermatozoa are smoothly rounded, with no trace of the acrosome except the filament (Fig. 10), although partially reacted sperm are frequently encountered. On supernumerary sperm the filament is always very short (1 to 1.5 μ), but in a few cases filaments of between 3 and 3.5 μ were produced on reaction with strong egg-water.

M. sulcataria (Spring to early autumn)

Spawning was induced by warming to 25° C. Reactive gametes were also obtained by cutting open the animals.

The spermatozoa of this species are similar in their proportions to those of *M. veneriformis*, but larger (Fig. 7). The acrosomal differentiation into the same three components as those of the *Mytilus* spermatozoan is clearly observable; the basal structure includes the greater part of the refringent acrosome substance, but a smaller amount lies separate from and distal to this.

The acrosome reaction was observed in supernumerary sperm only. These showed a short filament and the sperm head smoothly rounded anteriorly, as in *M. veneriformis* under the same conditions (Fig. 8).

Zirfaea subconstricta (Late spring)

These animals spawned in response to the combined warming (25° C.) and egg-suspension treatment. On insemination, nearly all the spermatozoa in the vicinity of an egg are found to have undergone the acrosome reaction (Figs. 14, 15), some remaining attached to the vitelline membrane by the relatively short (ca. 2 μ) acrosome filament, and others adhering to the coverglass by the distal part of the head (Fig. 16), indicating that the breakdown of the acrosome leaves the surface under it sticky (as in *Spondylus* and *Crassostrea*).

DISCUSSION

In the first study of this series, the term "acrosome breakdown" was used tentatively, even hesitantly, to describe a phenomenon which takes place in sea urchin spermatozoa on such a small scale that vital observation is virtually impossible. No structure is visible in the intact acrosome, the reaction itself cannot be observed in the intensely active spermatozoa, and all judgments concerning the steps in the process must be based on successively fixed preparations. These clearly show that some of the acrosome substance is lost from the anteriormost tip of the sperm head, leaving a slender filament, less than one micron in length. Whether this filament is projected beyond the original length of the acrosome, or is simply exposed by the dispersal of the surrounding substance, could not be determined. The fate of the acrosome membrane is also rather uncertain. Apparently the anterior part at least undergoes some sort of autolysis which stops before it quite reaches the base of the acrosome.

So far as the morphology of the structures involved in the acrosome reaction is concerned, the bivalve molluscs provide much more favorable material for study, since in some species (*e.g., Mytilus edulis*) the acrosome alone is longer than the combined head and middle piece of sea urchin spermatozoa, and considerable internal differentiation is easily visible in the living cells. In this group, moreover, exposure to the proper stimulus definitely results in a complete breakdown and dispersal of the acrosome, while the filament is clearly projected well beyond its original dimensions.

Among the species examined, the general structure of the head and acrosome is much the same throughout the group, although there is a considerable size range. Since the reaction process occurs similarly in all the species, it seems safe to generalize on the basis of observations made on the larger forms.

The differentiation of the acrosome into three regions has been described as it appears in the living *Mytilus* spermatozoan; this differentiation can be followed in the descending size scale through *Petricola* and *Spondylus* to *Mactra*. In four other genera—*Lithophaga, Chama, Zirfaea* and *Crassostrea*—only two regions can be seen, consisting of an axial structure passing through the hyaline acrosome substance; and in the very small acrosome of *Trapezium* no clear differentiation can be observed.[3]

Evidence which will be reported separately indicates that in *Mytilus*, the breakdown of the acrosome releases a substance which has a strongly lytic effect on the egg membrane. Since both the basal and distal parts of the acrosome break down together, it is impossible to localize the lysin in either of them on the basis of cases in which the reaction has occurred normally. However, the observation reported above of "partial reaction," in which the intact basal structure is associated with failure of filament extrusion, suggests that the mechanism which ejects the filament is located in this basal portion, and consequently, that the lysin is contained in the distal part of the acrosome.

"Partial reaction" in the spermatozoa of other species (*e.g.*, *Petricola* and *Lithophaga*) is also characterized by the persistence of an axial structure which is not longer than the intact acrosome, and is greater in diameter than the normally extruded filament. On the basis of these various observations, it is suggested that the axial structure consists of a tubular sheath, possibly enclosing some part or precursor of the filament.

In support of the conjecture that the basal part of the acrosome has the function of ejecting the filament, it would be gratifying to find that the length of the filament was correlated with the size of this basal structure in the other genera studied. This, however, is not the case. The acrosome of *Spondylus* is much smaller than that of *Mytilus* (*cf.* Figs. 1 and 11), but the *Spondylus* filament, measuring 15 μ on an average, is longer than the *Mytilus* filament. *Crassostrea,* also, has a still smaller acrosome but a relatively long filament. Such lack of correlation between the size of the basal part of the acrosome and the length of the filament does not preclude the possibility that this structure is concerned with the extrusion of the acrosome filament in bivalve molluscs, but it does emphasize the necessity for further investigation. Some other method besides phase contrast microscopic observation must also be used to determine whether the apparent absence of differentiation of the acrosome substance in the second group (*Lithophaga,* etc.) indicates a marked reduction in the amount of lysin, as compared with the first group, or a much smaller filament-ejecting apparatus.

This survey of the acrosome reaction in the bivalves, then, complements the echinoderm studies by confirming the generality of occurrence of both the acrosome filament and the substance believed to be the egg-membrane lysin, and by providing, within the limits of the common phenomenon, a scale of cases ranging from those in which the filament is more conspicuous to those in which the lysin seems more important. Until, however, this substance can be shown to be effective in dissolving the egg-membrane in a number of species, an inquiry into the possible reasons for its presence in larger or smaller amounts is premature.

Moreover, until more is known about the role of the acrosome filament in the fertilization process, it is difficult to identify the factors which determine its length. There does not seem to be a simple taxonomic relation involved, since long and short filaments are found in closely related genera (*e.g.*, *Mytilus* and *Lithophaga*). The idea that the filament serves to establish contact with the egg surface across a jelly layer which is impenetrable to the intact spermatozoan was suggested in the case of the starfish, but is entirely inapplicable to molluscan species having jellyless eggs, such as the oysters. Moreover, in other species, the intact spermatozoan has no difficulty in penetrating the jelly layer when one is present.

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[3] This may well be due to a failure of phase contrast resolution, since the acrosome is less than 0.5 μ in inside diameter, and its substance is highly refractive.

Undoubtedly the structural and chemical nature of the outermost layer of the unfertilized egg in each species is a most important factor in determining the characteristics of the acrosome which is charged with the task of breaching that particular barrier. Electron micrographs of the unfertilized *Arbacia* egg (McCulloch, 1952) show it to have an extremely thin vitelline membrane, which would be expected to yield quickly to the action of a small amount of a specific lysin. The oyster egg, on the other hand, is surrounded by a relatively thick, though apparently not tough, layer, to the outside of which the fertilizing spermatozoan remains so closely fixed as to seem fused with it for from two to four minutes, before slowly sinking through it into the egg.

In conclusion, it should be pointed out that this survey was undertaken primarily with the aim of extending the generality of the acrosome reaction, and makes no pretense of adequately sampling the Pelecypoda in a systematic sense. A few species were found in which the spermatozoa could not be caused to exhibit any reaction of the acrosome similar to that reported here, but in every case the basic morphology of such spermatozoa differs considerably from the fundamental plan characteristic of these twelve species. The spermatozoa of *Venerupis semidecussata,* for example, has a cone-shaped head like that of sea urchin sperm, with a long, slightly curved, sword-like process, greater in diameter than the sperm tail, in the position of the acrosome. There is some evidence that a filament contained within this process is exposed at the time of fertilization. The sperm head of *Cardita leana,* on the other hand, is extremely slender throughout its length, with a very small, sharply pointed acrosome in which no reaction of any sort has yet been detected.

There is every possibility that further work with these and other species will bring to light variations in the general pattern of acrosome structure in this molluscan group. Such studies must be correlated with an investigation of the finer structure of the surface layers in the unfertilized eggs before extensive generalizations can be made concerning the mechanism of sperm entrance in these forms.

The writers acknowledge their indebtedness and express their gratitude to the staff of the Misaki Marine Biological Station, and to the Tokyo Institute of Technology, for the use of the electron microscope.

SUMMARY

1. In twelve species of bivalve molluscs, it has been found that the spermatozoa undergo a reaction of the acrosome similar to that found among echinoderms. In all the species the spermatozoa react thus to the presence of unfertilized eggs; in some species egg-water and contact with glass surfaces are effective.

2. This reaction is characterized by the complete disappearance of the acrosome and the extrusion of a slender filament, the length of which varies considerably among the members of the group.

3. In the large *Mytilus* acrosome, three differentiated regions can be distinguished, consisting of a basal structure which seems to be concerned with the extrusion of the filament; a distal region containing what is believed to be an egg-membrane lysin; and an axial structure which appears to be a tubular sheath, possibly surrounding a precursor of the filament. The same regions can be seen in the spermatozoa of *Petricola*, *Spondylus* and *Mactra*. In the smaller acrosomes of *Lithophaga*, *Chama*, *Zirfaea* and *Crassostrea*, only two regions can be distinguished, and the acrosome of *Trapezium* is too small to show any clear differentiation.

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FREEZING IN INTERTIDAL ANIMALS[1]

J. W. KANWISHER

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Low temperature is frequently the chief environmental stress which eliminates a species in a given location. Marine life is generally exposed to a minimum of − 1.7° C., the freezing point of sea water. In the intertidal zone a much lower extreme must be met. On a high latitude shore in winter one finds an abundance of invertebrates exposed twice daily to a period of − 20° C. or lower. At these temperatures ice formation in the animals might be expected. This has been found to be the case and is the subject of this paper.

The observations on freezing in surviving macroscopic animals are scattered and frequently indefinite. The literature is reviewed by Luyet and Gehenio (1940) and more recently by Scholander *et al.* (1953). In spite of the large number of references only a few experiments are found in which quantitative ice measurements were made on surviving animals. Sacharov (1930) showed that as much as a third of the body water could be changed to ice in some insect larvae. More remarkable is the better than 90% frozen body water that Scholander measured in chironomid larvae. It seems likely that this large fraction of ice is present in the qualitative experiments reviewed by Luyet and Gehenio. These include the survival at liquid air temperatures of nematodes, rotifers, and tardigrades. Such forms would be expected to have a high water content.

In the Woods Hole region in winter the shore supports large numbers of mussels, snails, oysters, and barnacles. Many of these are high up on the shore where they are exposed to the air for six hours or more at low tide. They are frequently in completely exposed locations, where it seems they could not escape any adverse effects of the cold. The sea spray freezes on them and they may be imbedded in ice for days or even weeks at a time.

The rate of cooling of an animal can be estimated from the known values of heat conductivity in tissue and shell. This was done assuming that the animal is subjected to a sudden drop of 20°. Such is the case when it emerges from the water on the receding tide on a cold day. With even the larger mussels the temperature at the center will be within a degree of the air temperature after an hour's exposure.

To check these estimates the temperature of the interior of several molluscs was measured without moving the animals. For this purpose a small hole was drilled through the shell with the animal still in its natural location. A thermistor probe one mm. in diameter was inserted through this hole and could easily be pushed deep into the tissues.

Several such measurements were made on a day when the air temperature was − 15° C. The animals had been exposed for at least two hours by the receding tide. Specimens of *Crassostrea virginicus*, *Mytilus edulis*, and *Modiolus modiolus* showed without exception interior temperatures within a few tenths of a degree of the air temperature. There seems no reason to doubt that the smaller snails and barnacles are in the same condition.

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[1] Contribution No. 778 from the Woods Hole Oceanographic Institution.

Ice could also be felt with the thermistor probe. These animals were alive and appeared normal when brought into the laboratory. From this it appeared reasonable that internal ice formation can take place without being fatal to the animal. It was next undertaken to determine the amount of ice formed at various temperatures.

METHOD

Water, on crystallizing to ice, gives off heat and also expands. These two properties have been separately employed in determining the amount of ice in biological material. The dilatometer as used by Gortner (1937) and others measures the expansion directly. The flotation method of Scholander *et al.* (1953) determines the specific gravity change which results from this expansion. Both of these techniques require that the animal be free of gas bubbles. This seemed impossible to determine in an animal living in a shell, so it was necessary to use a calorimetric method.

When a frozen animal is introduced into the calorimeter a certain number of calories of heat are absorbed to raise its temperature to some value above 0°. Part of this represents the heat of fusion of any ice which may have been present in the animal. The rest is the result of the heat capacity of the animal and its shell. The specific heats of these components are uncertain so it is impossible to calculate the latter value. However, in this work it is measured directly by finding the calories necessary to warm the animal through a temperature range in which there is no ice formation.

An ordinary wide-mouth Thermos bottle proved to be an efficient calorimeter vessel. A mercury thermometer through the stopper was read to .01° with a lens. By varying the amount of water in the calorimeter different sized animals could be studied efficiently. The freezer compartment of a home refrigerator was used to cool the animals. By adding additional insulation the temperature variation during the cycling of the refrigerator thermostat was less than 0.25° C. To minimize heat loss the animal was transferred to the calorimeter at the door of the refrigerator with cold tongs.

The calorimeter was calibrated by dropping weighed amounts of ice into it. Small cups were pressed from aluminum foil. These were filled with fresh water and frozen to a known temperature. The amount of heat absorbed in warming one from a temperature T_1 below 0° C. to a temperature T_2 above 0 is equal to:

$$C = M_w(T_1 \times .49 + T_2) + M_w \times 79.6 + M_{al} \times .21(T_1 + T_2)$$

where: C = gram calories

M_w = mass of water

M_{al} = mass of aluminum cup

.49 = average specific heat of ice

.21 = specific heat of aluminum.

The heat capacity of the calorimeter is then computed from

$$\text{heat capacity} = \frac{C}{T} \text{ calories/degree}$$

where T is the temperature drop in the calorimeter vessel resulting from the introduction of the ice.

If the heat capacity of a specimen were constant with temperature a number of calories equal to $(T_2 + T_1)$ times its heat capacity would be required to warm it. All calories in excess of this could then be attributed to the change of state from ice to water. This is not strictly true since the specific heat of ice is half that of water. As more ice is formed the heat capacity of the animal is correspondingly lowered. This results in the correction shown in Figure 1. The rigorous equation of Ditman *et al.* (1942) is difficult to apply and in actual practice some of the terms are very small. The graphical method used here is believed to have an accuracy commensurate with the experimental technique.

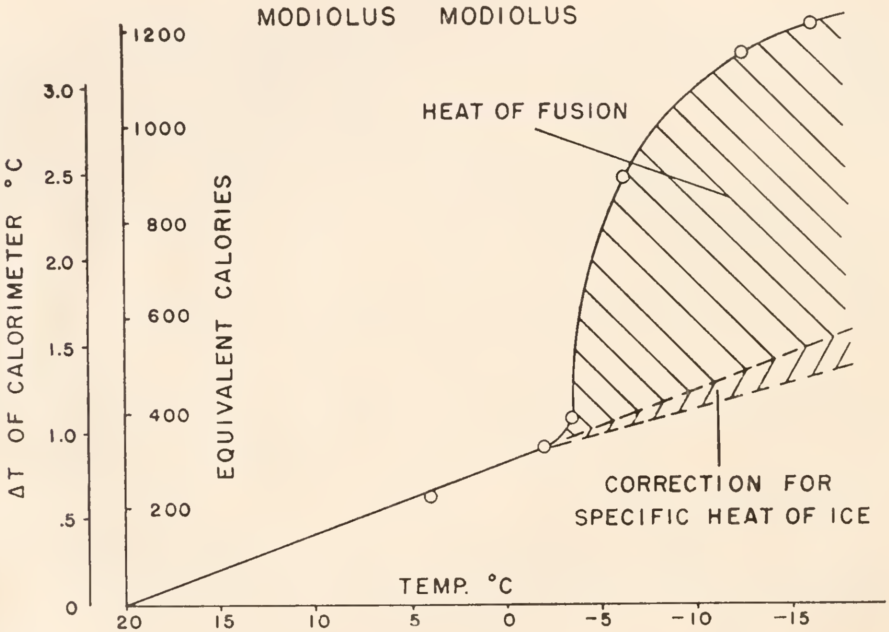

FIGURE 1. Graph showing ice formation in the mussel Modiolus modiolus. The shaded portion represents the calories used in the melting of ice. All points are from a single individual. The heat capacity of the animal changes as more ice is formed, resulting in the correction shown.

Ice determinations were made on animals frozen normally on the shore. They were placed directly in the calorimeter with a heavily gloved hand. Values obtained were essentially the same as for the animals frozen in the laboratory.

The bivalves were wedged open and all excess sea water forcibly shaken from them. They closed normally when the wedge was removed. They were then wrapped with a layer of self-vulcanizing rubber tape which provided a water-tight cover with a minimum of insulation. This cover allowed the animal to be removed from the calorimeter and refrozen at a different temperature without varying the amount of water in the shell.

In order to work with a single individual of the smaller species, such as Littorina, a method of micro calorimetry was worked out. The calorimeter vessel consisted of a round bulb on the end of a long thin-walled glass tube. Loose cotton insulation was packed around this. Such an arrangement was found to have a desirably small temperature drift. In a small calorimeter vessel the ratio of surface to volume becomes unfavorably large. It can be shown that most of the heat leakage is by way of conduction through the wall forming the opening to the chamber. To reduce this the thinnest glass tube possible was used. The methods employed on larger systems, such as silvering and operating in a vacuum, do not appreciably improve a small calorimeter. The volume of the bulb varied from 10 to 25 cc. depending on the size of the specimen. The temperature was measured with a thermistor suspended in the water. The same method of calibrating with a weighted amount of ice was used.

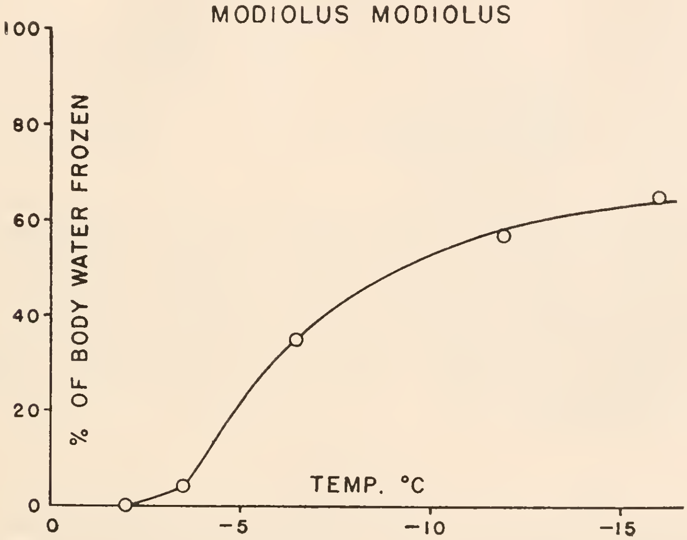

FIGURE 2. Graph showing the percentage of water as ice. The calories of the shaded portion of Figure 1 were converted to grams of ice and expressed as the percentage of the total water in the animal.

RESULTS

Figure 1 is typical of the results obtained. It is a graph of the amount of heat necessary to warm the animal up to 20° C. after it has been equilibrated for several hours at some lower temperature. Since the specific heats of tissue, shell, etc. are not appreciably altered by temperature one might expect a linear relationship. The amount of heat should depend only on the temperature range if the heat capacity of the system is unaltered. However, below 0° a larger number of calories than expected is absorbed. It is this additional heat that is required to melt the ice in the animal. Its amount can be found from the curve at different temperatures and converted to grams of ice. Since the total body water is found at the end of the run it is known what fraction of this is frozen at the various temperatures. Figure 2 is the result of such a calculation from the data in Figure 1. All points on a curve such as the one in Figure 1 are measured on a single animal. The data were not considered if the animal was not alive and of normal appearance at the end of the run.

TABLE I

Percentage of water frozen at —15° C.

Species	% frozen at −15°
Mytilus edulis	62
Modiolus modiolus	65
Littorina littorea	59
Crassostrea virginicus	54
Littorina rudis	67

Table I gives the fractions of body water as ice at − 15° C. in the different species examined. Individual measurements were made on Modiolus and *L. littorea* at − 22° C., the lower limit of the refrigeration equipment. The fractions of body water frozen at this temperature were 71% and 76%, respectively. The animals survived several days exposure to this temperature. *Littorina rudis* was studied at Hebron in Labrador while on an expedition sponsored by the Arctic Institute of North America.

DISCUSSION

Bachmetjew (1899), Salt (1950), and others have claimed super cooling of animals as great as − 15 to − 20° C. However, they expressed the strong belief that freezing was fatal and seemed to reason inversely that when the animal was dead it was therefore frozen. Ditman *et al.*'s (1942) calorimetric work represents one of the few quantitative ice studies. It was necessary for them to use a number of insects simultaneously in the calorimeter. They reasoned that the appearance of more and more ice at lower temperatures was due to variability in the super cooling of the individuals. Their curves indicate 70% frozen body water for coddling moth larvae at − 18°. This seems at variance with their conclusions of super cooling. This is not supplemented with measured lethal low temperatures although in a later paper Ditman shows complete survival of this species at this temperature.

The values in Table I show large amounts of ice at − 15° in all the forms that could stand freezing. The shape of the ice-temperature curve for the other species was the same as that in Figure 2. Some of the forms could stand a period at − 5° without ice formation. In no case, though, was this observed at − 7° or lower. Values of ice in the region of super cooling were obtained by cooling the animal to a lower temperature to start ice formation and then warming the animal. Super cooling of shore animals in nature only appears to occur down to −7° as a maximum.

As much as 75% of the water in the animals can be tied up as ice. It is interesting to speculate on the severity of the physiological stress represented by this sudden dehydration.

With the living processes in the protoplasm deprived of this water one might expect a slowing down of the metabolic rate. Lichens have been shown to respire at a rate related to their moisture content (Smyth, 1934; Neubauer, 1938). Scholander *et al.* (1953) have shown a precipitous drop in oxygen consumption concurrent with ice formation in a chironomid larva. This resulted in an apparent Q_{10} as high as 50 in this low temperature range.

In an animal that is 75% frozen the remaining brine concentration is 4 times the normal value. Actually some of the salts will probably have begun to precipitate out at this temperature and concentration. One might suspect many dire consequences at such a high salinity. Lovelock (1953) has published results which indicate that the high electrolyte concentration is the destructive factor in the freezing of red blood cells. The dehydration and high salinity appear to be factors the intertidal species can tolerate, at least at these low temperatures.

Ice is thought not to occur inside cells without damage (Chambers and Hale, 1932; Asahina *et al.*, 1954). It seems reasonable that such a finely organized structure as protoplasm could not stand the physical disruptions of intracellular ice. Apparently the cells are able to allow for a rapid exit of water and to stand the distortion of intercellular ice crystals. Meryman (1953) has shown this situation in slowly frozen rabbit liver. In the thawed tissue the water has migrated back into the cells and the appearance is once again normal. Siminovitch and Briggs (1952) have related frost hardiness in plants with an increased ability of the water to diffuse in and out of the cells. Asahina and collaborators have observed both extra- and intracellular freezing in insects. The latter was always fatal to the cells involved. Proof of such a situation in the frozen shore life can only come from histological examination.

The ice determinations here are reproducible within a range of ± 5%. Weighed amounts of ice could be measured with a 2% error. However, the large amounts of shell and wrapping hinder thermal equilibrium and require many calories for their warming. This forms a background above which ice determinations in such animals must be made.

In Figure 3 the freezing curve for a mussel is reproduced with one for sea water. This latter was obtained by treating a capsule of sea water in the same manner as the animals, *i.e.*, it was cooled to different temperatures and warmed in the calorimeter. No freezing curve for sea water could be found in the literature. The mussel contains significantly less ice than sea water at all temperatures. Part of this may be due to the lack of thermal equilibrium in the animal, particularly on the steep part of the curve. However, the many theories of bound water lend support to the idea that the disparity of these curves is real. To some extent the body fluids can be considered salt solutions and to this extent such a freezing curve would be expected. Any departure may well represent the participation of water in living processes other than as that of a simple solvent. Such a molecule as water with its high dipole moment can be held in low energy fashions unlike conventional bonding. This would compete with freezing which is itself a low energy per molecule process. When the physics of water in living systems is more thoroughly understood a closer approximation to the actual freezing curve can be theorized.

Attempts were made to freeze a large variety of molluscs, echinoderms, annelids, and arthropods which occur only below tide level. No animal was found to stand low temperatures and large internal ice formation that is not faced with these conditions in nature. The hardiness towards ice formation may be the principal reason allowing intertidal species to successfully invade the shore. Resistance to freezing runs parallel with the ability to withstand dehydration (Scarth, 1944; Siminovitch and Briggs, 1952; etc.). Both of these conditions are found in a situation of tidal exposure. The shore snails can be kept out of water for a period of weeks as can the mussels. However, all the forms frozen so far are encased in a shell and it is not certain how much natural dehydration they undergo independent of freezing.

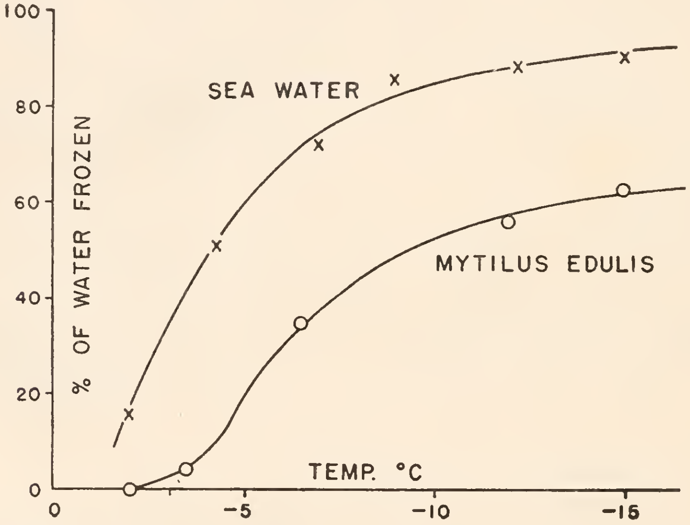

FIGURE 3. Graph of ice determined in the same fashion for a vial of sea water and a mussel. The difference is believed to represent unfreezable or bound water.

Finally, some observations were made in the Arctic that are interesting. In this region of permafrost the only protection from freezing afforded poikilotherms in the winter is in the sea. Hebron Fiord in Labrador has a year round bottom temperature of − 1.7° C. In spite of this the extensive bottom invertebrate fauna could not stand freezing. Here are animals that live and breed right up against the freezing barrier, and yet the particular adaptations of the shore life are absent. The only two plentiful intertidal forms were Mytilus and Littorina. Both of these displayed their usual resistance to freezing. Beds of Mytilus were noted which spend 6 to 8 months frozen solid in the ground ice at temperatures of − 20° C. and below! In the spring the Eskimos chip them out for food.

I am greatly indebted to Dr. Per Scholander for his continued interest and help-

ful advice during this work. My wife has also been of great assistance, particularly under the trying conditions of cold winter days on the shore.

SUMMARY

1. The temperature environment of shore life in the Woods Hole vicinity has been found to range as low as − 20° C. in the winter. *In situ* measurements have shown that no protection in the way of a micro climate is afforded most of this life.

2. A variety of intertidal forms has been frozen in nature and in the laboratory and their ice content measured. In live animals as much as 75% of the body water has been shown to be in the form of ice at temperatures regularly met in nature.

3. Some physiological consequences of this ice formation are considered.

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A VOLATILE FACTOR IN RELATION TO IN VITRO SPERMATOGENESIS IN THE CECROPIA SILKWORM[1]

MELVIN M. KETCHEL[2] AND CARROLL M. WILLIAMS

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

In one of the few successful applications of tissue culture techniques to insects, Schmidt and Williams (1953) were able to culture the spermatogonia and spermatocytes of diapausing silkworm pupae in simple hanging-drops of the insect's blood. The same technique in slightly modified form was subsequently used by Schneiderman, Ketchel and Williams (1953) in a study of the metabolism of spermatogenesis. These investigations, in brief, showed that dormant cysts of the male sex cells develop promptly into bundles of spermatids and spermatozoa when cultured in blood obtained from non-diapausing donors. Since comparable development failed to occur in cultures prepared in the blood of diapausing pupae, such blood was presumed to be deficient in a growth factor present in the blood during the non-diapausing stages. Indeed, it proved feasible to use the *in vitro* technique as a method of assay for this growth factor. The latter, on the basis of several lines of evidence, was considered to be the prothoracic gland hormone—the primary stimulus for cellular growth and metamorphosis within the insect as a whole.

In the course of the studies just considered, it was observed that cultures prepared in individual depression slides failed to develop and, in fact, underwent more or less prompt degeneration unless closed off from communication with the outside air. Moreover, in experiments utilizing larger culture chambers, it proved imperative, not only to seal the chambers tightly, but also to place a number of cultures in each chamber. Thus, an individual culture when isolated in a large sealed chamber under otherwise optimal conditions underwent far less development than when a number of other cultures were simultaneously present in the same chamber. This latter practice of placing numerous cultures in each chamber was routinely adopted by Schneiderman, Ketchel and Williams (1953) as an empirical solution of the problem. However, it was clear that the phenomenon was of sufficient interest in itself to merit further study. Consequently, we have returned to the initial problem in an effort to define the basis for the interaction of cultures in the same chamber.

MATERIALS AND METHODS

The experiments were performed on spermatocytal cysts obtained from diapausing pupae of the Cecropia silkworm according to the methods described by

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[2] Present address: Harvard University Blood Characterization and Preservation Laboratory, Jamaica Plain, Massachusetts.

Schneiderman, Ketchel and Williams (1953). Diapausing pupae served as donors of "inactive blood"; "active blood" was obtained from post-diapausing individuals on the first day of adult development. In order to block the tyrosinase reaction, all samples of blood were saturated with phenylthiourea.

Each culture chamber consisted of a brass ring (4″ in inside diameter and 0.08″ in thickness) sandwiched between a pair of glass plates (5″ × 5″ × 0.06″) and sealed together with melted paraffin. The plates had previously been given a hydrophobic coating with silicone ("Dryfilm 9987," General Electric Company). The chamber enclosed a gas volume of 17 cc. and could accommodate up to twenty sitting-drop cultures of approximately 20 $mm.^3$ each. In experiments utilizing gas mixtures, the brass ring was equipped with stainless steel inlet and outlet tubes as previously described.

In all save a few special cases a minimum of two such chambers were utilized in each experiment; namely, a control chamber containing 16 cultures in active blood, and an experimental chamber containing one or more cultures prepared from the same blood and cyst suspension. After seven days at 25° C., 30 to 40 per cent of the cysts in the control cultures had ordinarily progressed to Stage III (spermatidal cysts) or IV (spermatozoal cysts). The experiment was terminated at this point and the degree of development in each chamber ascertained. For this purpose the number of cysts which had developed to Stages III or IV was recorded by counting a total of 500 cysts in a series of randomly selected low-power fields. Ordinarily the response in the control cultures was taken as 100 per cent, and the response in the experimental cultures calculated as a percentage of that of the controls.

INFLUENCE OF THE NUMBER OF CULTURES PER CHAMBER

The quantitative effects of the number of cultures enclosed in each sealed chamber were studied in detail in two series of experiments. In the first experiment a suspension of cysts was prepared in active blood and distributed among five chambers so that the number of cultures per chamber ranged from 0.5 to 8. Seven days later, counts were made of 500 cysts in each chamber and the percentage of the 500 in Stages III or IV recorded.

The summary of results in Table I shows that chambers containing fewer than 4 cultures supported the development of a small proportion of cysts. The response was enhanced in the 4-culture chamber and further enhanced in the 8-culture chamber.

TABLE I

The number of cultures in each sealed chamber conditions the developmental response

Cultures per chamber	Per cent response* Expt. 1	Expt. 2
16	—	45
8	21	43
4	14	12
2	8	15
1	2	13
½	4	—

* Per cent of the total cysts counted which had reached Stage III (spermatidal cysts) and Stage IV (spermatozoal cysts) after 7 days of culture.

The second experiment (Table I) utilized a cyst suspension which showed a greater degree of response in all preparations. Minimal development was found in the 1, 2, and 4-culture chambers. By contrast, the 8- and 16-culture chambers showed a three-fold increase in the proportion of cysts attaining Stages III or IV.

On the basis of numerous other experiments comparing the development attained in 1-culture and 16-culture chambers of the volume described above, we can state that the difference was never less than three-fold and commonly much greater than three-fold.

Absence of Stimulation by Non-Growing Cultures

The experiments just considered indicate that the development of the cysts in any one culture is promoted by the simultaneous presence of other cultures in the same sealed chamber. It is therefore worth inquiring whether this same influence can be exerted by non-growing cultures prepared in inactive blood, or, indeed, by drops of active or inactive blood in the total absence of cysts.

To test these possibilities, 16 cultures of a cyst suspension in active blood were placed in a control chamber, and one culture of the same suspension in each of four experimental chambers. Three of the experimental chambers then received, respectively, 15 drops of a cyst suspension in inactive blood, or 15 drops of active blood without cysts, or 15 drops of inactive blood without cysts.

The results computed as per cent of the response of the control cultures are recorded in Figure 1. It is obvious that none of these maneuvers served to increase the low level of response of the individual test cultures. Consequently, it is clear that the agency by which one culture interacts with another is generated only by cultures capable of growth; *i.e.,* by cultures containing both cysts and active blood.

Hormonal Requirement

Since the development of spermatocytes was already known to require the presence of the hormonal component of active blood, it seemed possible that the developmental factor derived from actively growing cultures might correspond to some volatile derivative of this same hormone. Under this circumstance a culture prepared in inactive blood should develop if enclosed in the same chamber with numerous cultures in active blood.

An experiment of this type was performed, one culture in inactive blood being placed in the same culture chamber with 16 cultures prepared in active blood. The results of this experiment were clear-cut and decisive: the cultures in active blood developed promptly, whereas no development occurred in the test culture in inactive blood. This finding demonstrates that the stimulating factor produced by developing spermatocytes cannot substitute for the hormone, and that the presence of the hormone is prerequisite for the response of spermatocytes to the stimulating factor.

Effects of Carbon Dioxide and Oxygen Tensions

It will be recalled, as mentioned in the introduction, that a single culture in the limited volume of a depression slide develops normally provided that the hormone is present and the cover-slip tightly sealed. But if the cover-slip is placed slightly ajar, then no development occurs and the cysts promptly die. This fact, in itself, directs attention to the gaseous medium within the sealed chamber and its critical modification by the developing culture contained therein. Evidently, in the case of the larger chambers utilized in the present study, a considerable number of cultures are required to "condition" the greatly enhanced gas volume.

The problem at this point seemed extremely simple since a basis for conditioning the gaseous medium was obviously present in the respiration of the cysts themselves.

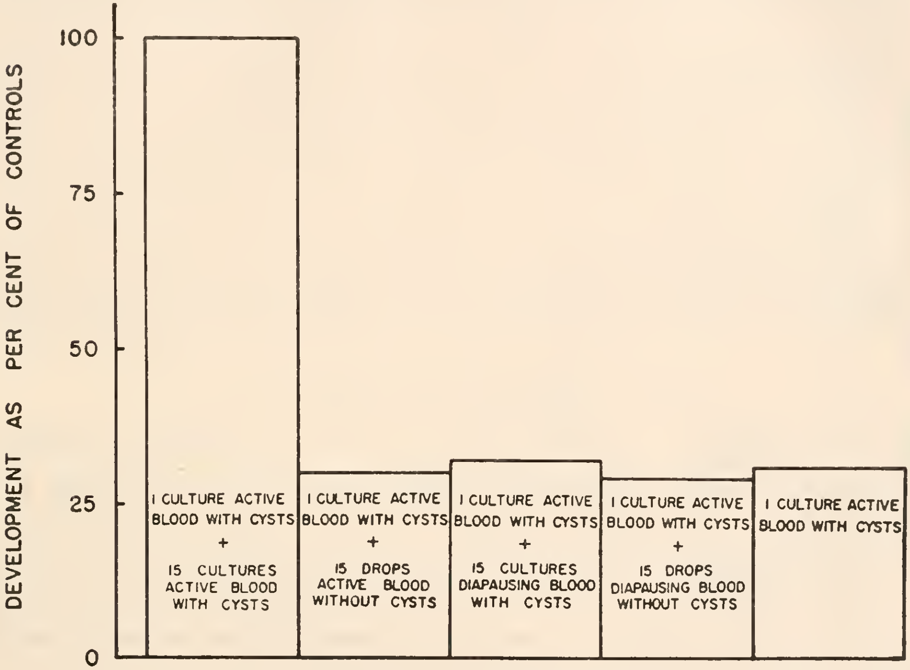

FIGURE 1. Interaction between individual cultures in the same chamber. The growth response of the test culture is enhanced by the presence of cultures containing spermatocytes in active blood, but is not enhanced if either of these components is absent.

In order to test whether the stimulating factor might correspond to a critical tension of carbon dioxide, two cultures of cysts in active blood were placed in one chamber and 16 cultures in each of two other chambers. One of the latter chambers then received, in addition, 16 drops of 0.1 *M* potassium hydroxide for the purpose of absorbing carbon dioxide. Seven days later the 2-culture control chamber showed the expected low percentage of development, while the 16-culture control chamber showed the anticipated high percentage. Of present interest and importance is the fact that the same high percentage of development was observed in the chamber containing the 16 cultures plus alkali. This experiment was repeated with the same uniform result. Consequently, a critical tension of carbon dioxide cannot be the stimulating factor.

The possibility that a critically low oxygen tension was the source of stimulation was a far less attractive hypothesis, since Schneiderman, Ketchel and Williams (1953) had already demonstrated that the *in vitro* development of spermatocytes was independent of oxygen tensions ranging from 1 to 21 per cent of one atmosphere. This finding was re-examined in the following experiment.

A series of mixtures of oxygen and nitrogen were prepared containing 20, 15, 10, 5, and 0.5 per cent oxygen, respectively. Two cultures of cysts in active blood were placed in each of five experimental chambers and 16 cultures in a sixth chamber that served as a control. Each experimental chamber was flushed for five minutes daily with one of the above-mentioned gas mixtures in order to establish and maintain a particular oxygen tension. Each experiment was performed in duplicate.

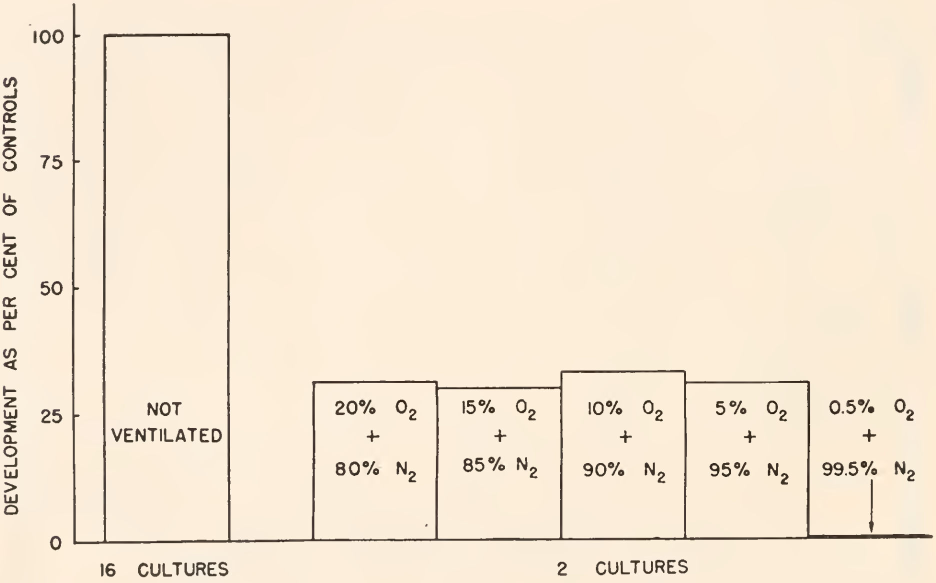

FIGURE 2. Daily ventilation with gas mixtures containing specific low oxygen tensions fails to enhance the developmental response of the two cultures in each of a series of five chambers. The experiment was performed in duplicate series.

The results recorded in Figure 2 show that the developmental response was independent of oxygen at tensions ranging from 5 to 20 per cent, whereas in 0.5 per cent oxygen development was completely blocked. Consequently, the stimulating factor can scarcely correspond to a critical oxygen tension established by respiration.

CONDITIONING OF THE CULTURE MEDIUM

Though unresolved at this point, the "conditioning" of the gaseous medium is reminiscent of the conditioning of the culture medium which, in the case of vertebrate tissue cultures, is thought to precede the growth response (Parker, 1950). As is now generally recognized, the cells of vertebrates must first react with the culture medium to establish a critical concentration of some unknown factor before growth becomes possible. Consequently, the establishment of a culture from a single vertebrate cell was successfully accomplished only after Sanford, Earle and Likely (1948) developed the technique of initiating such cultures in a capillary tube containing a minute volume of medium. In order to determine whether, in the case of spermatocytes, a conditioning of the culture medium precedes the conditioning of the gaseous medium, the following experiment was performed.

A cyst suspension was prepared in active blood and distributed as two cultures in each of four chambers. The residual cyst suspension was then diluted with seven volumes of the same sample of active blood; the diluted suspension was distributed as 16 cultures in each of four chambers. Consequently, all chambers contained the same total number of cysts (*ca.* 800), whereas the volume of culture medium differed by eight-fold in the two series. It was reasoned that if the same number of cysts developed in all chambers, then any influence of the conditioning of the medium could be disregarded. Alternatively, if development was enhanced in the more concentrated cultures, then the conditioning of the culture medium must be taken into account.

The answer provided by the experiment was neither of these possibilities, but yet a third; namely, an average of 36 per cent of the cysts developed in the more dilute suspensions whereas only 10 per cent developed in the concentrated ones. Consequently, under the conditions of the experiment, the presence of the larger volume of blood per cyst resulted in the greater response.

THE VOLATILE FACTOR

1. Effects of ventilation

A suspension of cysts in active blood was distributed among eight chambers, two cultures being placed in each of four chambers, and eight cultures in each of four. All eight chambers were immediately flushed for five minutes with a gas mixture containing 20 per cent oxygen and 80 per cent nitrogen. This same flushing procedure was repeated daily for two of the four chambers of each series, the other chambers remaining sealed as controls.

As recorded in Figure 3, the results were clear-cut in that development in the ventilated cultures was greatly depressed. Indeed, development in the ventilated 8-culture chambers was depressed virtually to the level observed in unventilated 2-culture chambers.

2. Solubilities of the factor

Eight cultures in active blood were placed in each of a series of chambers along with 8 to 10 drops of various solvents; namely, 0.16 M HCl, 0.16 M KOH, 0.1 M H_2SO_4, 0.32 M sucrose, light mineral oil, or inactive blood. None of these solvents was able to depress development when tested in this manner. Consequently, the experimental procedure was modified to increase the surface area of the various solvents. For this purpose each chamber was subdivided by a barrier consisting of a folded strip of filter paper, and the latter saturated with solvent. Sixteen cultures in active blood were placed on one side of the barrier and one culture on the other side. In this manner, any volatile agent generated on the "donor" side of

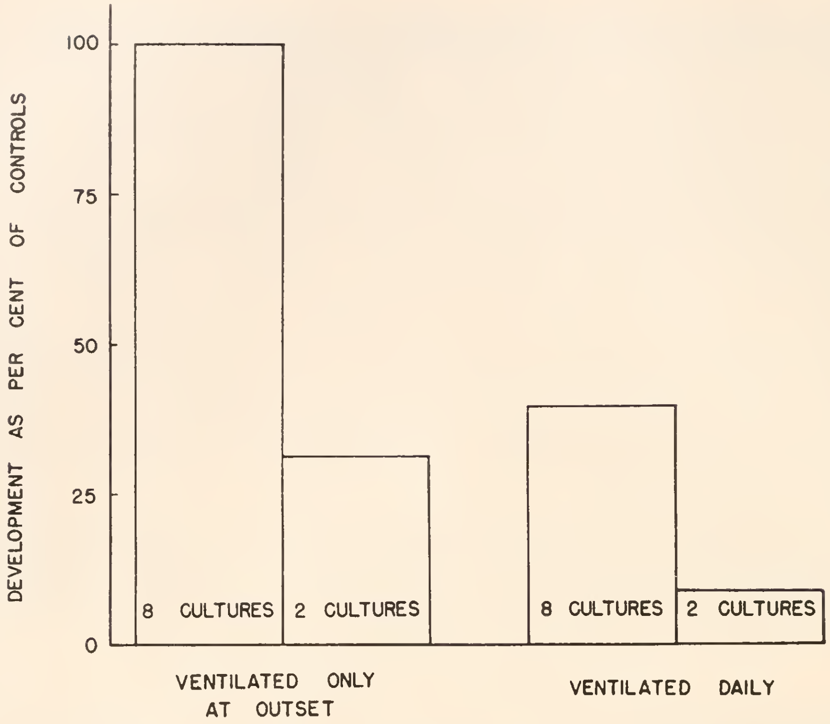

FIGURE 3. The daily ventilation of the chambers with air inhibits the developmental response. For further explanation, see text.

the chamber was forced to traverse the broad surface of solvent before reaching the recipient drop on the other side.

The results summarized in Table II show that acid, base, sugar solution, and Ringer's solution were ineffective when tested. However, mineral oil now produced a slight but definite inhibition. Of special interest and importance is the fact that inactive blood, spread upon the filter paper, considerably reduced the development on both sides of the chamber. Indeed, the development of the re-

TABLE II

The per cent of cysts developing in a single culture separated from fifteen other cultures by a barrier moistened with one of the solvents noted

Solvent	Per cent development
Insect Ringers solution	34
0.16 M NCl	37
0.16 M KOH	34
0.1 M H_2SO_4	32
0.32 M sucrose	35
Light mineral oil	24
Inactive blood	0 (cysts darkened and swollen)

cipient drop was totally suppressed and the cysts therein soon became dark, swollen, and obviously dead.

3. Adsorption on activated charcoal

As diagrammed in Figure 4, 16 cultures in active blood were distributed in three rows on one side of each of four chambers, and on the other side was placed 0.5 to 1 gm. of activated charcoal ("Norit A"). A fifth chamber lacking charcoal was used as a control. In the row of cultures nearest to the charcoal, development was completely suppressed: within twenty-four hours all the cysts in this row were swollen, dark, and dead. The cysts in the intermediate row of cultures appeared to remain viable, but only 2 per cent developed. In the row of drops furthest from the charcoal, the cysts remained viable and 14 per cent developed. In the control culture lacking charcoal, 42 per cent of the cysts developed.

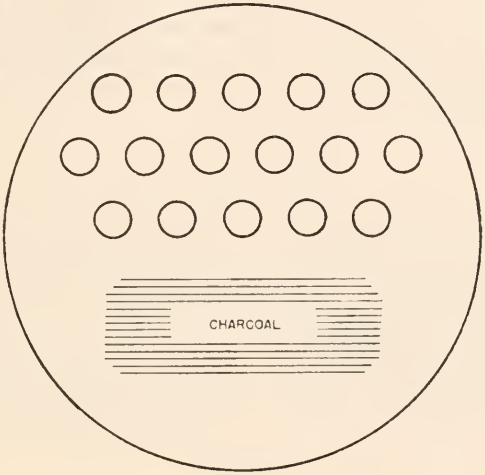

FIGURE 4. Semi-diagrammatic representation of the arrangement of cultures and charcoal in test chambers.

The experiment was repeated using a fresh cyst suspension and a second sample of Norit A from an unbroken package. The same results were obtained.

In order to eliminate the possibility that the charcoal released some toxic volatile principle, control chambers were ventilated with a current of air drawn through a column of charcoal and then sealed. Normal development was observed in all cases.

We therefore conclude that the volatile agent is adsorbed by activated charcoal. Evidently, in the above-mentioned experiments the adsorption was limited by the diffusion of the factor across the chamber to the charcoal from its site of production in the cultures. The most distant cultures preserved a sufficient local concentration of the agent to permit survival and a limited developmental response. In cultures closest to the charcoal, the concentration of factor was necessarily minimal.

It is of particular interest that such cultures not only failed to develop, but also underwent abnormal changes leading to death.

4. Effects of charcoal on cultures in inactive blood

The experiment described in the preceding section and diagrammed in Figure 4 was repeated utilizing a cyst suspension in inactive blood. A similarly prepared chamber without charcoal served as a control. Because of the sub-threshold titer of hormone in the culture medium, no development was expected and none was observed. Special attention was centered on the microscopic appearance of the cysts as a criterion of their viability. After seven days, the two rows of cultures furthest from the charcoal remained normal in all respects and could not be differentiated from the controls. By contrast, the cysts in the row nearest to the charcoal showed slight darkening and an abnormal degree of granularity. In the absence of appropriate tests, we cannot state with certainty whether these cultures remained viable. However, there could be little doubt that their abnormal characteristics were far less prominent than previously noted for equivalent cultures in the presence of hormone.

Discussion

The experimental results indicate that the development of spermatocytes requires the presence, not only of hormone, but also of a further developmental factor which the hormone-stimulated cells generate within the culture medium. This factor is soluble in blood and volatile. Consequently, it distributes itself between the culture medium and the surrounding gas phase. The amount of factor which a single culture can generate is limited, but can apparently be enhanced by increasing the volume of blood medium available per cyst. This suggests that the cysts generate the volatile factor from a non-volatile precursor within the medium and that it is this precursor which is limiting.

Though hormonally stimulated cells die in the absence of the volatile factor, among the population of cysts in any culture one observes a broad distribution of thresholds for stimulation by the factor. A single culture can equilibrate with the scanty gas volume of a sealed depression slide and still retain within itself a sufficient concentration of the factor to stimulate a high proportion of cysts. However, in larger chambers having an internal volume of 17 ml., eight cultures are required to establish and maintain an equivalent concentration. Hence, it may be calculated that a single culture can equilibrate with a gas volume of about 2 ml. before the concentration of the factor begins to limit the response of the cysts with high thresholds.

By virtue of the circumstances just described, individual cultures are protected against excessive loss of the volatile factor by the presence of other cultures which are likewise generating the factor. Consequently, the individual cultures interact *via* the gas phase which they share.

Though the chemical nature of the volatile factor is a matter for further investigation, the information already at hand permits certain inferences. The volatility, in itself, indicates that the factor is neither a protein nor any other very large molecule. Moreover, its failure to react with acid or alkali is suggestive of a neutral molecule. The absence of detectable solubility in aqueous solvents means that the factor is neither hydrophilic nor strongly polar. Its definite, though limited, solubility in mineral oil indicates the presence of apolar groups. Though insect blood contains about 85 per cent water, the properties of blood as a solvent are complex and poorly understood; consequently, little can be inferred from the high solubility of the factor in this medium.

It is a curious fact that the present phenomenon most closely parallels the action of ethylene in relation to the ripening of fruit. As recently reviewed by Biale (1950), there is now general agreement among plant physiologists that unripe fruits, under the over-all stimulation of auxin, generate within themselves catalytic amounts of ethylene; the latter is then essential for the biochemical events associated with ripening. A single apple is said to produce a total of up to one cc. of ethylene gas, while experimentally one finds ethylene to be effective in concentrations as low as a few parts per million.

On the distant chance that ethylene might likewise be involved in the "ripening" of the insect sex cells, the gas was tested in several low concentrations and found inactive as a substitute for the volatile factor. However, it is not unlikely that the techniques developed by botanists in collecting and identifying ethylene may prove helpful in further study of the present problem. The testes of the intact insect receive an intimate tracheal supply, and it is probable that the volatile factor escapes from the tracheal system during the period of adult development. Presumably this loss is steadily replaced by a transformation of the precursor present in the blood.

Though the developmental factors in insect spermatocytes, as in the fruit of plants, could scarcely have been detected had they not been volatile, it is our opinion that volatility is a property only incidentally associated with the molecules in question. Of far greater interest is the demonstration that the reaction between cells and hormones can generate molecules which are catalytically active and prerequisite for the biological end-result.

SUMMARY

1. The *in vitro* development of spermatocytes of the Cecropia silkworm is inhibited when individual cultures are placed in large chambers rather than in depression slides. The inhibition is relieved by increasing the number of cultures in each sealed chamber—a finding which demonstrates that the individual cultures interact.

2. The basis of this interaction was studied in detail. Evidence was obtained that the interaction occurs *via* the gas phase within the chamber by virtue of the production by the cultures of a developmental factor which is volatile.

3. The role of this volatile factor was studied in relation to *in vitro* spermatogenesis. Its production was detected only in cultures containing spermatocytes and hormone. These components along with the volatile factor were then found necessary for the developmental response.

4. By suitable tests it was possible to show that the volatile factor was neither carbon dioxide nor any other normal constituent of air.

5. The factor is definitely soluble in insect blood, slightly soluble in mineral oil, and relatively insoluble in water. On the basis of present evidence, it appears to be a neutral, apolar molecule.

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DIFFERENCES IN INTENSITY OF SETTING OF OYSTERS AND STARFISH

V. L. LOOSANOFF, J. B. ENGLE[1] AND C. A. NOMEJKO

U. S. Fish and Wildlife Service, Milford, Conn.

The ecological association of the American oyster, *Crassostrea virginica* Gmelin, and the common starfish, *Asterias forbesi* Desor, is well known. The latter, in many areas, is the chief enemy of the former. These common representatives of the two phyla are not only found living under the same conditions, but also resemble each other in their methods of reproduction. They are both diecious and oviparous, discharging eggs directly in the sea water where fertilization takes place. Spawning in both species occurs during the same season, although the starfish usually begin approximately two weeks earlier. The lengths of the spawning and setting seasons of both species are of approximately the same duration, the latter, in Long Island Sound, extending in some years from early July until the first week of October (Loosanoff, 1936; Galtsoff and Loosanoff, 1939; Loosanoff, 1937–1954).

A comparison of the reproduction of these two species could be carried even further because both produce a large number of eggs from which small planktotrophic larvae develop. After a relatively long pelagic period of two to three weeks, the larvae of both species descend to the bottom where they metamorphose into juvenile oysters or starfish. This step in the development of these organisms is usually known as setting and the recently metamorphosed individuals are called either starfish or oyster set.

Regardless of the similarity of the reproductive behavior of these two invertebrates, and even though their larvae develop during the same season and under the same general ecological conditions, such as temperature, salinity, pH, turbidity, currents, winds, etc., their intensity of setting in relation to each other shows a complete absence of any definite pattern. This conclusion is based on our observations of reproduction in these two species in Long Island Sound during the 18-year period extending from 1937 through 1954. Brief summaries of these observations, together with the analysis of the data on which we have based our conclusions, are presented in this article.

The method used in our studies consisted of counting, at regular intervals, the numbers of oyster and starfish sets found on the collectors. These collectors were wire-mesh bags filled with clean oyster shells. The bags were anchored on the bottom and buoyed in a special manner described by Loosanoff and Engle (1940). The collectors, all of uniform size and shape, and containing approximately the same number of shells, were placed at stations situated along the Connecticut shore of Long Island Sound in the area extending from the Bridgeport to the New Haven harbors, a distance of approximately 30 miles. The stations were established at

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[1] Presently of Chesapeake Shellfish Investigations, Annapolis, Maryland.

10-, 20-, and 30-foot depths and their number in the different years varied from seven to 18. Since 1944, however, the number of stations has always been ten and their locations have remained the same.

At semi-weekly intervals the collectors were removed from the stations and brought in moist condition to the laboratory for examination. Using a low-power dissecting microscope the oyster set was counted on ten shells selected at random from each bag, and then the same ten shells, plus an additional ten shells, were examined for the presence of starfish set. The results were later expressed as the number of set of each species per 100 shell-surfaces. Counting of set was done only on the clean inside surfaces of the shells.

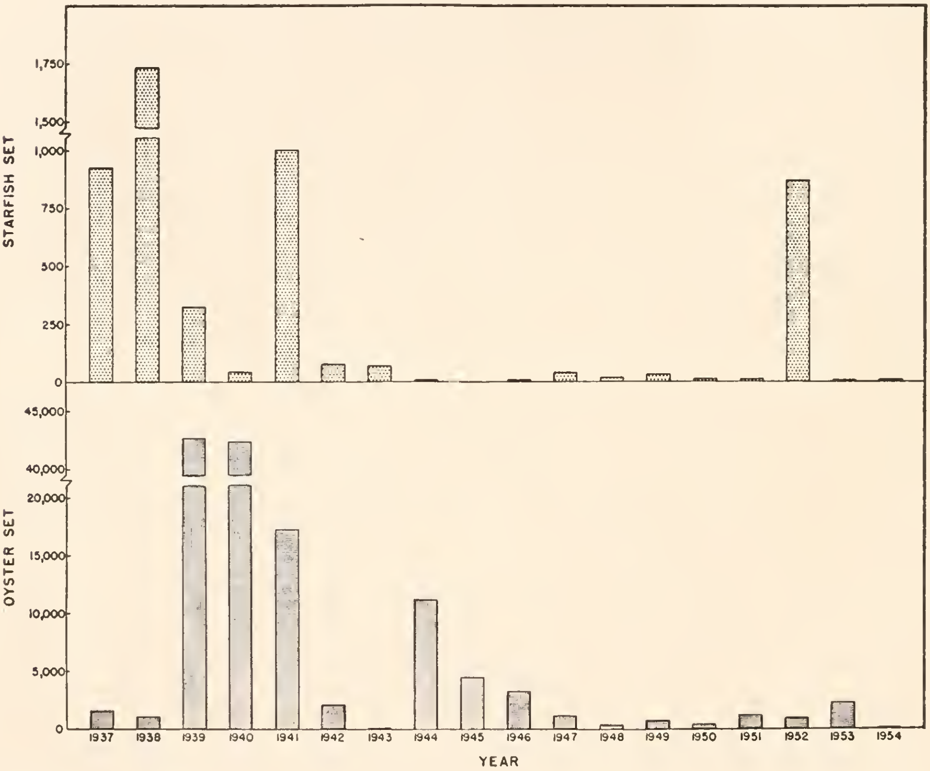

FIGURE 1. Average numbers of oyster and starfish sets per station recorded in different years on 100 shell-surfaces. Long Island Sound, 1937–1954.

Each time a bag was removed from the water it was immediately replaced by a new one with unused shells. To obtain more reliable data we used, as a rule, two bags at each station, and averaged the results. The period during which the observations were made usually extended from the beginning of July into October, covering, therefore, the entire setting season of both species.

The intensities of the oyster and starfish sets in the different years are shown in Figure 1. They are averages based on the number of set found during the entire summer on the inside surfaces of 100 shells at each station. As can be seen, during the 18-year period the oyster set was usually heavier than that of the starfish. The only exception was 1938 when the average seasonal starfish set per station was 1731 per 100 shell-surfaces, as compared with 1090 for the oysters. The year of maximum starfish set for the entire period of our observations was also 1938, while the year of heaviest oyster set was 1939 when 42,623 spat per station was the season's average. As the other extreme, we may mention that in 1945 only one recently-set starfish was found on our collectors during the entire summer, while the lightest oyster set occurred in 1943 when the average seasonal set was only 72 spat per station per 100 shell-surfaces.

To study further the discrepancies in the setting of the two species, product-moment correlations between the oyster and starfish sets were computed with the set for each year expressed as a percentage of that of the year when the highest setting took place. Thus, 1939 was taken as the 100 per cent year for oysters, and 1938, for starfish. This approach gave a range for each variable of 0 to 100 per cent. In each case, however, the distribution was badly skewed with most of the years falling between 0 and 10 per cent.

To make the analysis more detailed, correlations were determined for the stations located at each of three depths and also for the combinations of stations at several depths.

All correlations obtained were very low:

For 10-foot stations only $r = .06$
For 20-foot stations only $r = -.05$
For 30-foot stations only $r = .03$
For 10- and 20-foot stations $r = -.02$
For 10-, 20-, and 30-foot stations $r = -.01$

With N, number of years in this case, being 18, the product-moment correlation (r) must be at least .47 to be significant at the .05 level. The low correlations obtained indicated that there was no definite relationship between the oyster and starfish sets. However, since the distribution of the data was badly skewed, we thought that the use of the conventional product-moment correlation coefficient might give somewhat misleading results. To verify the conclusions we decided to use rank-order correlations (r_s) also. For this, the yearly setting data of each species were assigned ranks from 1 to 18, in order of magnitude, and the correlations were then computed according to the rank correlation coefficient formula. The coefficients obtained were as follows:

For 10-foot stations only $r_s = .19$
For 20-foot stations only $r_s = .03$
For 30-foot stations only $r_s = .06$
For 10- and 20-foot stations $r_s = .07$
For 10-, 20-, and 30-foot stations $r_s = .02$

Since the value of the rank-order correlation coefficient necessary for significance at the .05 level, with $N = 18$, is .47, all correlations obtained were not significant.

The absence of correlation was also well illustrated by scatter plots of the oyster set against the starfish set. A plot showing the lack of relationship between the rank-order of the set of the two species at the 10-, 20-, and 30-foot stations all combined in one group, is given in Figure 2. As may be seen from this plot, and from the low coefficients for each depth and combination of depths, almost complete dissociation is suggested between the variables. Therefore, we concluded that there is no appreciable relationship between the intensities of the starfish and oyster sets for the years and depth-stations for which data were available.

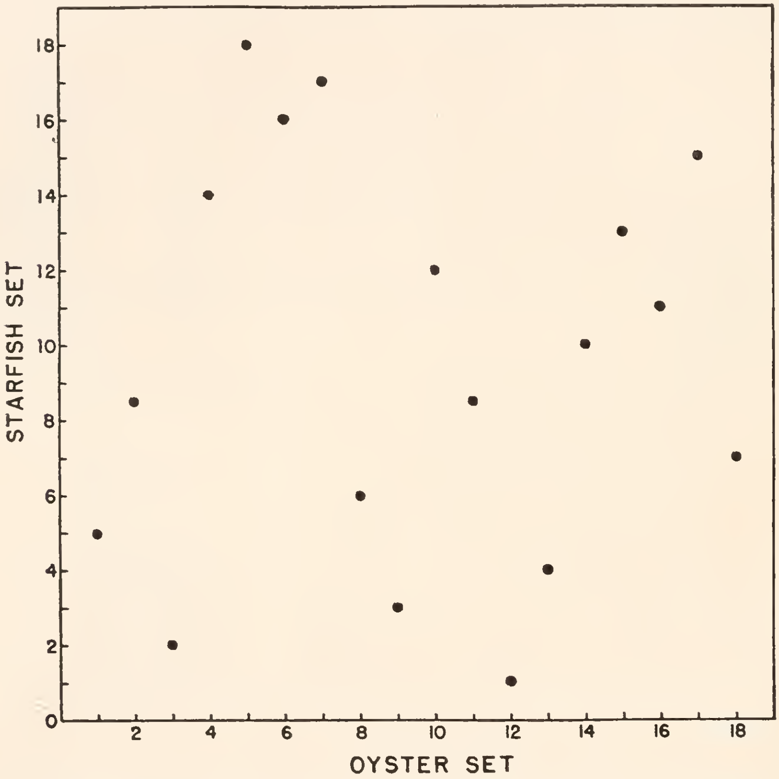

Figure 2. Rank-order relationship of oyster and starfish sets at 10-, 20-, and 30-foot stations. Long Island Sound, 1937–1954.

These studies, conducted for almost two decades, emphasize once more the danger of forming conclusions regarding the relationship between certain natural phenomena, if such conclusions are based on relatively short periods of observations. For example, if only the first four years of our period of observations were considered, a highly significant negative correlation between the intensity of the oyster and starfish setting would be indicated (Fig. 1). This, obviously, could lead to serious misconceptions. In our case, fortunately, by continuing the studies for many more years a much truer understanding of the situation was made possible.

The question naturally arises as to the causes responsible for the absence of a well-defined pattern in the intensity of setting of the two species in relation to each other. At present we are not prepared to discuss this question at length. Nevertheless, as mentioned above, we know that adult oysters and starfish live and propagate under the same ecological conditions, and that their larvae, which are present in the water during the same season, are subjected to the same general sets of environmental factors. We may suppose, therefore, that unfavorable conditions, such as relatively low summer temperature, strong winds, etc., would affect the larvae of both species more or less equally. Because oyster and starfish larvae are microscopic in size and both pelagic, we may also assume that most of the predators of one would also be feeding upon the other.

Low salinity, conversely, might be suspected as a factor which could unfavorably affect starfish larvae while leaving oyster larvae relatively unaffected. It is known that even adult starfish cannot survive in a salinity lower than 16.0 parts per thousand (Loosanoff, 1945), while oysters can normally live and reproduce in water of much lower salinity. However, in Long Island Sound proper, where our studies have been conducted, the salinity in summer is steadily near 27.0 parts per thousand, thus being within the optimum range for both starfish and oysters. Consequently, this ecological factor cannot be considered, under our conditions, as important in favoring one or the other species.

The density of the parent populations in different years is another factor to consider. Our observations on these populations (systematic surveys of the occurrence and distribution of starfish are made twice a year and oysters are collected near our stations at weekly intervals during the summer) showed that never during the period of our studies was the density of either population so reduced as to be below the minimal. In general, the starfish population of Long Island Sound remained large, as attested by our surveys and also by the extensive and continuous efforts of the oyster growers combatting these pests. The oysters, although reduced in number because of poor sets during the past seven or eight years, and the heavy mortality caused by the storm of 1950, are still numerous enough to be counted by the hundred thousand bushels. For example, we know that on the beds of a single oyster farming company in the Bridgeport area, there are now approximately 150,000 bushels of two-year-old oysters. Considering the unusually high fecundity of the oyster and the comparatively high fecundity of the starfish we believe that there are still sufficiently large parent populations of both species to produce enough spawn so that, under favorable conditions for survival and growth of larvae, heavy sets could result. This was well demonstrated for starfish during the summer of 1952 (Fig. 1). Since we know this and since our other studies, to be reported elsewhere, have shown that there is no direct relationship between the numbers of spawners available and the intensity of setting of oysters or starfish in Long Island Sound, we may conclude that the fluctuations in parent populations were not the principal causes responsible for the lack of relationship observed between the intensity of setting of the two species during our 18-year studies.

Although at this time we lack data to demonstrate it, an absence of relationship in the intensity of setting of oyster and starfish larvae may be due to only one or to a few causes of rather specific nature. For example, there is little doubt that the larvae of the two species may exhibit different food requirements, as far as size and quality of food organisms are concerned. Thorson (1946) says that an advanced echinoderm larva can take in particles as large as 50 μ, while our observations have shown that even "eyed" oyster larvae, about ready to metamorphose, require food of much smaller size, probably not over 15 μ in diameter. Moreover, since it has already been demonstrated that the larvae of even such closely related species as oysters and clams display widely different food requirements (Loosanoff, 1950; Davis, 1953), it is clear that even those food forms that are small enough to be taken in by both species may not be assimilated by one of them. Consequently, the presence or absence of such specific food organisms may be primarily responsible for the survival and, therefore, intensity of setting of larval oysters or starfish.

Another possibility to be considered is what we call, for lack of a better name, "water factor", which probably indicates the presence in sea water of certain dissolved substances that are needed for the normal existence and development of certain marine animals. These aspects of sea water chemistry and of animal behavior are still not well understood but we have evidence indicating that under laboratory conditions the presence or absence of this, at present, undetermined substance, or substances, may affect the growth of larvae of one species while not affecting the larvae of another closely related form (Davis, 1953).

Finally, only recently has the attention of biologists been called to diseases and parasites of lamellibranch larvae (Davis *et al.*, 1954; Loosanoff, 1954). It is possible, then, that larvae of other marine invertebrates, such as starfish, may also be victims of attack of different pathogenic micro-organisms, and that these micro-organisms may affect only closely related groups. If this is true, such organisms may sometimes eliminate the larval population of one species without harming the other. As a result, although most of the other conditions of the environment would be equally favorable to larvae of both forms, the larvae of the species suffering the epidemic would fail to set. Perhaps pathogens, together with specific food requirements of the larvae and the role of the water factor in their existence, explain in part the lack of relationship in the intensity of setting of oysters and starfish in Long Island Sound.

Since in the history of marine biology there are only a few studies when simultaneous systematic observations of the reproductive behavior of two ecologically closely associated species have been conducted steadily in the same area for long periods, we hope that the information offered in this article will contribute to a better understanding of the fluctuation in the natural populations of marine invertebrates existing in the same habitat.

We wish to extend our thanks to Barbara Myers for the statistical analysis of the data used in this article and to Rita Riccio for helping prepare the manuscript.

SUMMARY

1. No consistent relationship was found between the intensities of the oyster and starfish sets of Long Island Sound during the 18-year period, 1937-1954.

2. It is suggested that specific larval diseases, specific food requirements and presence or absence in the water of certain, at present, undetermined substances,

may be responsible, to a large extent, for the fluctuations in the intensity of setting of the two species in different years.

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SYNERGISM AND ANTAGONISM IN THE INDUCTION OF METAMORPHOSIS OF BUGULA LARVAE BY NEUTRAL RED DYE[1]

WILLIAM F. LYNCH

St. Ambrose College, Davenport, Iowa, and the Marine Biological Laboratory, Woods Hole, Mass.

It is well known in the literature of biology that two agents which influence protoplasmic systems in the same way may cause an enhanced effect when employed simultaneously. Synergism had been observed in previous work on *Bugula* larvae when neutral red dye was added to magnesium-free sea water. Both dye and magnesium-free sea water acting singly induced precocious metamorphosis, and their combined effects were greater than that of either acting along (Lynch, 1952). Antagonism between two factors, also, had been encountered. As previously reported (Lynch, 1947), temperatures of 7–8° C. had been found to inhibit metamorphosis in *B. neritina*. Subsequent experiments on *B. flabellata* showed that the larvae of this species failed to metamorphose during a forty-eight hour period of observation when kept in sea water in a refrigerator at 5° C., whereas at room temperature (24–27° C.) nearly all the organisms had undergone fixation within twenty-four hours. (In twelve observations the total percentage of unmetamorphosed larvae at room temperature was found to be 4%. The normal duration of the natatory period of larvae is discussed in another paper: *cf.* Lynch, 1952, p. 371.) In other experiments the inhibition caused by low temperatures persisted even when neutral red dye was added in proportions which, at room temperature, induced a rapid rate of setting. The four experiments to be discussed were performed on the larvae of *B. flabellata*. They were devised partly to test for synergism or antagonism in the induction of metamorphosis by neutral red in combination with four other factors, each studied separately: light, mechanical agitation, an anaesthetic and acidified sea water. But the observations were made largely to test a working hypothesis of metamorphosis suggested in a former paper (Lynch, 1952). The *modus operandi* of the dye, and especially the influence of anaesthetics and acidity on its action, form an integral part of the proposed explanation of metamorphosis, its induction and suppression by physical and chemical agents.

According to this tentative hypothesis, artificially induced metamorphosis in bryozoan larvae seems to be essentially a response to stimulation involving changes in viscosity of the protoplasm of the larvae, the exact site of these changes being unknown. The types of agents found effective for inducing metamorphosis belong to the general class of stimulants. There is a growing mass of evidence, stemming

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largely from the school of Heilbrunn and his co-workers, that the artificial initiation of cell division of an egg (without sperm) is a kind of stimulation. It seems to be more than merely coincidental that many agents which induce metamorphosis also cause artificial parthenogenesis in marine eggs. On the other hand, the inhibition of metamorphosis of *Bugula* larvae appears to be a kind of anaesthesia involving viscosity changes opposite to those which induce setting. This hypothesis is based largely on the effects of low temperatures (4° to 12° C.), of magnesium and potassium ions and of calcium-free sea water in preventing metamorphosis. Anaesthesia, according to Heilbrunn's theory of calcium release, ensues when such agents as ether and chloroform release calcium from the cortex of cells but prevent its binding with the inner protoplasm and thereby result in its liquefaction. Stimulation, on the other hand, occurs when agents release calcium from what seems to be a lipoprotein binding in the outer region of a cell and the free calcium enters the interior, becomes bound to the inner protoplasm and causes a clotting reaction essentially like that which occurs in blood (*cf.* Heilbrunn, 1952, pp. 604–613). Since stimulation cannot occur in the absence of calcium, the "colloidal theory" seems to explain the failure of larvae to metamorphose in calcium-free sea water. Furthermore, magnesium is a well-known depressant and both this ion and potassium have been found by Heilbrunn (1932) to have an anaesthetic effect on amebae. And finally, since the viscosity of some types of protoplasm (notably *Cumingia* eggs) is low at 2° C., gradually rising to a maximum at 15° C. (Heilbrunn, 1927), it seems plausible to assume that the inhibitors of metamorphosis all have a common effect—*viz.*, a lowering of viscosity. (It should be noted, however, that the viscosity curves of other types of protoplasm are not like those for *Cumingia*. Thornton, 1935, found the viscosity of *Amoeba proteus* to be low at 3° C., maximum at 7° C. and to decrease gradually again up to 36° C.; *cf.*, also, Costello, 1934.)

The first set of experiments was set up to test the anaesthesia hypothesis of inhibition of metamorphosis by using two well-known anaesthetics, alcohol and potassium cyanide. The interaction between alcohol and neutral red was somewhat incidental to the main purpose of the experiment. A second phase of the experiment with potassium cyanide involved a test for possible accelerating effects on metamorphosis following a removal of the larvae from the cyanide solution. This was considered worthwhile, since Loeb (1913) had reported parthenogenetic development of *Arbacia* eggs, previously activated to membrane formation by acid treatment, when the eggs were removed from sea water containing potassium cyanide; and LeFevre (1948), also, had noted similar effects on *Nereis* eggs whose sensitization by picric acid was enhanced by cyanide. Thus, the experiments with cyanide were devised to determine the effects of both the presence and the removal of this anaesthetic on bryozoan larvae.

The second set of experiments concerns the effects of mechanical agitation on metamorphosis. Observations made by the writer on *B. neritina* (Lynch, 1947) made it seem probable that prolonging the activity of the larvae by mechanical agitation inhibited metamorphosis in this species, as had been reported by Rogick (1939) for some fresh-water bryozoans. On the other hand, mechanical agitation generally acts as a stimulating agent causing liquefaction of the cortex of cells followed by gelation of the interior protoplasm (Heilbrunn, 1952, pp. 368, 607; Angerer, 1936). Furthermore, mechanical agitation had been reported by Grave

(1935) as a factor inducing metamorphosis in ascidians; and shaking is a well-known parthenogenetic agent for starfish (Matthews, 1901), for *Urechis* (Hiraiwa and Kawamura, 1936) and as a synergist with heat (but not with chemicals) in the activation of *Nereis* eggs (LeFevre, 1945). One would expect, consequently, that mechanical agitation either would have no effect on bryozoan metamorphosis, just as it has no effect on many types of marine eggs, or that it would hasten rather than retard setting. The effects of mechanical agitation, therefore, needed clarification. Again, the use of neutral red was somewhat incidental to the main purpose of the experiment. The dye was used merely to hasten setting so that significant counts could be made more quickly after mechanical agitation. The chief point at issue was to determine whether shaking would affect the rate of setting of the larvae. Thus, the question could be answered by noting synergism, antagonism or no correlation between dye and mechanical agitation.

The third set of experiments concerns the interaction of light and dye. Since darkness inhibits metamorphosis (Lynch, 1949a) and a moderate amount of diffuse light hastens setting (Grave, 1930), it appeared likely that neutral red would show a photodynamic action in the induction of metamorphosis, especially since many dye-stuffs have their effects enhanced by light (*cf.*, for instance, Tennent, 1937; Alsup, 1941; Blum, 1941).

The fourth set of experiments was devised to study the effects of acidity on larval motility and on metamorphosis and the interaction between acidified sea water and neutral red. Since other observations had shown that the dye actually enters the larval tissues and colors them visibly (Lynch, 1952), it seemed plausible to suppose that the dye molecules might induce metamorphosis by replacing calcium from protein-binding on the alkaline side of the isoelectric point and that the released calcium would induce protoplasmic clotting. According to this viewpoint, a cationic dye like neutral red should not have the same effect both above and below the isoelectric point of larval protoplasm. Furthermore, it had been found that metamorphosis did not occur below a pH of 5.8 and that the inductive action of an excess of isotonic calcium chloride in sea water was greatly reduced by a low pH (Lynch, 1952). Secondly, since some types of eggs, notably those of the starfish, are stimulated to undergo parthenogenesis when removed from acidified sea water (Lillie, 1926), it seemed worthwhile to have data on the effects of a similar removal of larvae from sea water having a low pH. Finally, it was necessary to re-examine the effects of acidity on metamorphosis, especially from the standpoint of possible independent effects of buffers. This had not been done in previous studies on the effects of acidity on metamorphosis.

Materials and Methods

As in previous experiments, the adult colonies were kept covered during the night before the observations were to be made; on the next day larvae were obtained by placing the parental colonies in a large finger bowl and exposing them to light. The photopositive larvae were readily pipetted within an hour from the region of the dish nearest the window. In all experiments with neutral red dye the writer used a 0.1% aqueous solution, obtained from the stockroom of the Marine Biological Laboratory. The proportions of dye added to sea water are given in parts by weight in the various tables, a lesser concentration having been used when it was disadvantageous to have the larvae metamorphose too rapidly. Aside from the observations on photodynamic action, both control and experimental groups received the same number of foot candles of light (determined by a Weston photometer), in order to eliminate this factor as a variable known to be significant. Both groups of larvae were also placed close together in stender dishes, so that temperatures would not vary; the range for all experiments was 24–27° C., except for those on the effects of potassium cyanide, performed at temperatures from 22–24° C. for reasons to be discussed later.

Counting was facilitated by using paper ruled into squares 5 × 5 mm., somewhat like the base of a haemacytometer except for size. Larvae stained red by dye were most easily counted by using white paper ruled into squares with black ink; unstained larvae showed best against black paper with squares made by white ink. Three or four counts were made for each dish to insure accuracy. Since a binocular microscope, which gives a field sufficiently large for counting, does not clearly reveal whether the larvae have metamorphosed or are only quiescent (or perhaps cytolyzed), the stender dishes had to be moved gently back and forth; attached larvae would stick rigidly, whereas unmetamorphosed quiescent ones would not. Larvae which became attached to the surface film were the most difficult ones to distinguish from those which were merely quiescent in this region of the dishes. The writer found that counting could be done most accurately by placing experimental and control larvae in a refrigerator about fifteen minutes before the counts were made; the low temperature (5° C.) activated quiescent larvae so that they could be distinguished easily from non-motile metamorphosed ones attached to the surface film. This was done in the experiments with potassium cyanide, actually the last ones performed in chronological order.

For the experiments on the effects of anaesthetics, the writer used absolute ethyl alcohol, at a pH of 7.8, and 0.001 *M* potassium cyanide in sea water, dropped from pH of 8.7 to 7.8 by HCl. Since trial experiments had shown that the slight hypertonicity of the cyanide solutions had no accelerating effects on metamorphosis, a characteristic of solutions having greater than normal osmotic pressure, no adjustment to isotonicity was considered necessary.

For the effects of mechanical agitation, shaking was accomplished by a motor-driven agitator producing back and forth movements in a horizontal plane at the rate of about fifty oscillations per minute. Both experimental and control larvae were exposed to the same concentration of dye, 1 : 300,000 in sea water ($pH = 7.8$), and to the same amount of light. After fifty minutes of agitation both groups were removed and counts were made ten minutes later, after the larvae had time to become quiescent. It was found that shaking for more than fifty minutes caused fragmentation of the larvae.

In the various sets of experiments on photodynamic action both experimental and control larvae were placed in sea water containing dye in proportions of 1 : 100,000. (A lower concentration of dye was used for experiments on mechanical agitation to prevent a too rapid setting of the controls while the experimental larvae were exposed to conditions that would probably not allow them to attach.) One stender dish was kept in darkness in each experiment, while the other was exposed to fifty foot candles of diffuse daylight, except in four instances in which the larvae received seventy-five foot candles. Several sets of experiments, preceding those on photodynamic action, had shown that there was no statistically significant difference in the rate of metamorphosis within a range of twenty to seventy-five foot candles.

Experiments on the effects of pH were carried out within a range of 4.5, 5.5, 6.5 and 7.8 by using glacial acetic acid, hydrochloric acid and acetate buffer as indicated in Table III. Observations on the effects of a removal of larvae from sea water acidified by glacial acetic acid were made at two different points on the pH scale, 4.5 and 5.4. Larvae were removed from the former after intervals of 1, 2, 3, 4, 5 and 60 minutes and from the latter after 30 minutes of exposure. For determining the interaction between acidity and neutral red, sea water containing dye in proportions of 1 : 300,000 was prepared at three pH values: 7.8, 6.5 and 5.5 Both HCl and acetate buffer were used, as indicated in Table III to drop the pH below that of normal sea water. Determinations of the hydronium ion concentration were made by a Beckman meter after the larvae had been seeded. To test for possible independent action of buffers, the following were used: acetate, borate, glycine, and both potassium and sodium McIlvaine buffers. (McIlvaine buffers are mixtures of citric acid and disodium phosphate; special buffers were prepared by substituting the dipotassium for the disodium salt.)

RESULTS

A. The effect of anaesthetics

Alcohol. Experiment number 7 of Table I is the most significant of the group, since it shows that alcohol can completely inhibit metamorphosis. The other experiments show antagonistic action between alcohol and neutral red dye. (1) The effect of alcohol is rather easily suppressed by the higher of the two concentrations of neutral red, 1 : 100,000, a 3.3% solution of alcohol and sea water giving some inhibition of metamorphosis by three hours but very little by twenty-

TABLE I

The effects of absolute ethyl alcohol alone and in combination with neutral red dye

No. exp.	Concentr. neutral red/sea water	Concentr. alchool/ sea water	No. larvae metamorphosed in hours		Inhibition by alcohol	Development of zooids	
			3	24		Exp.	Control
1	1:100,000	1.0%	++++	++++	slight	good	good
2	1:100,000	3.0%	++++	++++	slight	good	good
3	1:100,000	3.3%	+++	++++	slight	good	good
4	1:300,000	1.0%	++++	++++	slight	—	—
5*	1:300,000	3.0%	+	++	notable	poor	fair
6*	1:300,000	3.0%	–	++	notable	poor	good
7	none	3.3%	–	–	complete	none	—

* In exp. 5 and 6 there were more than the usual number of unmetamorphosed larvae at 3 hrs. for this concentration of neutral red and sea water.

++++ = *ca.* 90–98%
+++ = *ca.* 60–80%
++ = *ca.* 10–25%
+ = *ca.* 3–5%
– = none

The percentages are approximate. The pH of exp. no. 7 characteristic of the group, dropped from 7.8 to 7.5 within 24 hrs. Control larvae were in the same concentration of dye and sea water but without alcohol.

four. (2) With a concentration of 3% alcohol and that of the dye reduced to 1 : 300,000, (experiment 6) none of the larvae had metamorphosed by three hours and only about 20% had attached by twenty-four hours. (3) When the concentration of alcohol was raised to 3.3% and no dye was used, metamorphosis was inhibited completely within the twenty-four hour period of observation. This was found to be true of other experiments with the same concentration of alcohol. (4) If one compares experiments 3 and 7, it is evident that, when no dye is present (experiment 7), a 3.3% solution of alcohol and sea water suppresses metamorphosis, whereas the same concentration of anaesthetic is practically ineffectual when the concentration of dye is high (experiment 3). Larvae under the anaesthetic effect of alcohol show neither positive nor negative reactions to light, gathering in the center of the stender dishes, exactly like those subjected to

Table II

The effects of KCN (A), mechanical agitation (B) and photodynamic action (C) on the rate of metamorphosis of Bugula flabellata

A. Effects of KCN on the rate of metamorphosis

Experimental			Controls		
Larvae exposed for 30 min. to sea water containing 0.001 M KCN at a pH of 7.8 (from 8.7 by HCl). Larvae were counted 30 min. after removal from test solution.			Control larvae were pipetted to sea water and counted 60 min. later, at the same time as the experimental larvae.		
No. larvae	No. metamorphosed	Per cent. metamorphosed	No. larvae	No. metamorphosed	Per cent metamorphosed
109	9	8	189	82	43
30	21	70	161	85	53
69	6	9	157	98	62
12	6	50	102	42	41
59	3	5	23	17	74
47	0	0	46	41	89
63	4	6	33	26	79
389	49	12.6	711	391	54.9

Statistics

	Experimental	Controls
No. larvae	389	711
No. observations	7	7
Total number metamorphosed	49	391
Total per cent metamorphosed	12.6	54.9
Mean per cent	21.1	63.0
Standard deviation	27.3	19.2

$t = 3.13$; $P = .02$

F (variance for column means) $= 7.3$; $P = .03$

F (variance for row means) $= 0.29$; $P = > .10$

Note: the t value is calculated for a difference of two means of independent (uncorrelated) samples, since $r = -0.018$; $t = .03$. No correlation was found for observations made on the same day. Otherwise $F = t^2$ and $t = 2.7$; $P = .03$. The analysis of variance was made by the method of Croxton (1953, p. 295).

TABLE II—*Continued*

B. The combined effects of mechanical agitation and neutral red dye. Both experimental and control larvae were in sea water containing dye in concentration of 1:300,000 and at a pH of 7.8. Both groups were equally illuminated.

	Experimental			Control		
Larvae in dye solution and agitated for 50 min.				Larvae in dye solution and not agitated		
No. larvae	824			1189		
No. observations	15			15		
Rate of metamorphosis in hrs.	1	2	3	1	2	3
Total no. metamorphosed	217	656	800	430	964	1144
Total per cent metamorphosed	26.2	79.6	97.1	36.1	80.4	96.2
Mean per cent	29.2	71.6	90.6	43.2	83.5	91.8
Standard deviation	20.2	19.1	13.7	20.2	16.8	14.4

t for 1 hr. = 1.9; P = .08
t for 2 hrs. = 1.7; P = .10
t for 3 hrs. = 0.6; P = .50 or greater

The t value is calculated for the difference of two means of independent samples.

C. Combined effects of light and neutral red dye on the rate of metamorphosis. The dye concentration for both experimental and control larvae was 1:100,000 and the pH was 7.8.

	Experimental			Control		
Larvae exposed to 50–75 ft. candles of diffuse daylight				Larvae in dye solution and in darkness		
No. larvae	623			825		
No. observations	16			16		
Rate of metamorphosis in hrs.	1	2	3	1	2	3
Total no. metamorphosed	561	608	623	602	670	776
Total per cent metamorphosed	90.0	97.9	100	72.9	81.2	94.1
Mean per cent	90.0	98.8	100	71.6	85.0	96.3
Standard deviation	9.4	3.2	0	20.9	14.3	5.1

t for 1 hr. = 3.31; P = < .005
t for 2 hrs. = 3.68; P = < .005
t for 3 hrs. = 3.12; P = < .01

an excess of either magnesium or potassium chlorides in sea water (Lynch, 1949a).

Potassium cyanide. Larvae left in 0.001 M solution of potassium cyanide and sea water showed marked inhibition of metamorphosis when compared with the controls, but this concentration of cyanide did not completely prevent metamorphosis. In an experiment, typical of the others, 10% of the larvae attached to the surface film and developed into well-formed zooids; the remainder were geopositive during the observation, and of these organisms 20% metamorphosed forming zooids of retarded growth. The better development of larvae attaching to the surface film is characteristic of this organism, having been observed hundreds of times in experiments with other types of agents. Since Table II A shows the inhibiting effects of potassium cyanide after the larvae were removed from this solution to sea water, no actual counts were made on larvae left in the cyanide-sea water except the one given above. The experiments are easily reproducible, and the inhibiting effects of cyanide are patently evident when one compares experimental and control organisms after eight hours of exposure.

The unusually large number of control larvae metamorphosed by two and a half hours after their emergence from the ovicells, as indicated in Table II A, offered a challenging problem. Early setting was a characteristic of the larvae studied during the summer of 1954, when the observations on potassium cyanide were made. These experiments were carried out in a room where the air temperature often reached 27° to 29° C. by mid-afternoon, whereas the other experiments reported in this paper were performed in a cooler basement room (24°–27° C.) during the summer of 1952. Despite repeated attempts towards a solution of the problem by keeping the larvae of both experimental and control groups in a sea tank (22°–24° C.), where the temperature was actually somewhat lower than in previous years, the phenomenon of early setting continued. If higher temperatures were the cause of the abbreviated natatory period, the heat must have affected the organisms before they were shed as larvae. Since the adult colonies were exposed for several hours during the afternoons preceding each experiment, the heat may have caused the larvae to be shed in a state of more advanced development than in other years. A phenomenon apparently akin to the above occurs in some of the tunicates, which, according to Berrill (1930), may actually metamorphose before hatching. As pointed out by Wright (1934), development is the result of a large number of chemical and physical reactions, the rates and durations of which are determined by the history of the organism prior to the stage in question, by correlative reactions within the organism, by external environmental factors and by the action of the genes within each cell. The problem of variability in the natatory period of bryozoan larvae under normal conditions remains a puzzling one, as does that of epidemics of early settings which occur on certain days every year, despite the apparent sameness of the environment.

Table II A shows that the anaesthetic effects of cyanide could be detected 30 minutes after removal of the larvae from the inhibiting solutions to sea water. The difference in numbers of metamorphosed larvae in the two groups can be explained partly on a time basis alone, since some of the controls were metamorphosing during the thirty minutes when the experimental larvae were inhibited; but part of the differences seems to be attributable to a persistence of the anaesthetic effect even after the removal of inhibition. In this respect, bryozoan larvae do not react like those marine eggs that are stimulated to undergo cell division parthenogenetically after removal from cyanide solutions to sea water.

B. *The effects of mechanical agitation*

Table II B shows that only at the end of an hour was there a difference that was almost significant (P = .08) between larvae that were agitated and the controls. Since there was no difference in the two groups at the end of two and at the end of three hours, shaking apparently had no effect on the condition of the protoplasm of the larvae but caused a purely mechanical interference with setting.

a process that requires a certain degree of quiescence of organisms about to attach. Since both experimental and control groups were exposed to very dim daylight (less than ten foot candles) during the first fifty minutes of immersion in the dye, the percentage of metamorphosed larvae at the end of an hour was relatively small. (At 50 to 75 foot candles, as determined by nineteen sets of independent experiments on 1462 larvae divided into two groups, 67% of one group and 70% of the other had metamorphosed by the end of an hour in the same concentration of dye and sea water as was used for the observations recorded above.)

C. Evidence for photodynamic action

Table II C gives data for sixteen experiments with larvae immersed in sea water containing neutral red in proportions of 1 : 100,000 under conditions of light and darkness. The t ratios obtained, 3.31, for the difference of the two groups at the end of one hour and 3.68 at the end of two hours, show that the experimental larvae differed significantly at the 1% confidence level from the controls not exposed to light. The differences in the two groups became more apparent after an hour, when the dye had time to penetrate the larvae and bring about the changes that induce metamorphosis. These data afford ample evidence of photodynamic action. For some unknown reason, larvae that metamorphosed in darkness formed somewhat larger and better zooids at the end of eight hours than those which attached in the light.

D. The effects of acidity

Larval motility and the pH of the medium. Since it had been noted in experiments already published (Lynch, 1949a) that larval movements cease below a pH of 6.0, the following observations on motility concern only contrasting effects of constant versus variable pH.

When the stender dishes were covered to maintain a constant pH, the larvae remained almost motionless and no excursive swimming movements could be detected a few minutes after immersion. When the dishes were left uncovered, however, and the pH shifted upwards, the larvae began movements between a pH of 6.2 and 6.8. Larvae, first subjected to a low pH, continued to swim much beyond their usual period when either the pH was allowed to rise gradually or the organisms were transferred to normal sea water. Four experiments, typical of others, were recorded. In two instances involving a gradual rise of pH from 5.8 to 7.0–7.4 nearly all the larvae were still active at twenty-four hours, whereas less than 10% of the controls were motile. In another there were 24% swimming at the end of thirty-five hours and some were still active at forty-eight. Such prolonged swimming has not been observed in normal sea water. In a fourth experiment the larvae were transferred to their normal medium from sea water acidified to a pH of 5.5 by glacial acetic acid. Of these, 25% were still motile at twenty-four hours as opposed to only 4% of the controls. This persistent effect of acidified sea water in retarding metamorphosis after the inhibiting agent has been removed resembles that of potassium cyanide. The lower range for swimming movements of *Bugula* larvae corresponds fairly well to the values given by Rogers (1938) for ciliary action in other organisms, from 5.5 to 6.0. The upper limit in alkaline sea water has not yet been determined for these larvae. The motility of *Bugula* larvae is not affected by adding neutral red to acidified sea water.

Effects on metamorphosis. Larvae did not metamorphose when they remained in sea water acidified by glacial acetic acid to a pH of 4.5 or 5.5. As had been noted in experiments with other acids (Lynch, 1949a), these observations and others set the lower limit of pH ranges within which metamorphosis can occur at about 6.0. Larvae exposed to acidified sea water shed their outer ciliated covering, revealing denuded jelly-like remains, presumably the internal sac containing adhesive fluid. Nearly all larvae had reached this stage of complete denudation by eight hours. Cytolysis, nevertheless, was not as rapid as one might expect. At a pH of 4.5 to 5.6 disintegration was fairly extensive within three hours; but within a pH range of 5.6 to 6.2 the larvae maintained their integrity for eight to twelve hours. These organisms, therefore, are neither as resistant to acid cytolysis as *Nereis* eggs, which can withstand exposures to sea water acidified to a pH of 6.6 by picric acid for as long as seventy-two hours without injury (LeFevre, 1945), nor are they as susceptible as starfish eggs which are damaged by a slight overexposure to acid sea water (Lillie, 1926).

Transfer experiments. Transferring larvae after intervals of 1, 2, 3, 4, 5 and 60 minutes from sea water acidified to a pH of 4.5 by glacial acetic acid (one drop per 125 cc.) did not induce precocious metamorphosis. In fact, larvae that were exposed for 60 minutes to acidified sea water remained quiescent, except for ciliary movements, from the time of removal to their normal medium and did not metamorphose at all. Those exposed for one to 5 minutes, on the other hand, eventually metamorphosed, but the number of natatory larvae was larger than that of the controls, when compared at eight hours, and they were more vigorous in their swimming movements. Removal to sea water, therefore, had much the same effect as allowing the pH to rise gradually. Actual counts were not made, since the experiments are readily reproducible, and the difference in behavior of experimental and control larvae was clearly evident.

Inhibition of the action of neutral red by acidity. Table III shows a significant difference at the 5% confidence level ($t = 2.4$) in the effectiveness of the dye (1:300,000) at a pH of 7.8 as contrasted with a pH of 6.5. To test for persistence of acid effects, the pH was allowed to rise slightly, since dishes in all experiments except the last were uncovered. Note the greater inhibition of the dye by a low pH in the covered dish.

At a pH of 5.5–5.8 a dye concentration of either 1:300,000 or 1:100,000 failed to induce metamorphosis. Antagonism between dye and acidity was complete within this range, provided that the hydronium ion concentration was maintained at this level for twenty-four hours. This was done by covering the dishes, which prevented a rise of more than 0.4–0.5 of a pH unit during a twenty-four hour period. (The exclusion of oxygen will not prevent metamorphosis, for setting will occur in tightly corked bottles having only 20 cc. of undissolved air available.) The reduced effectiveness of a cationic dye such as neutral red in acid media is in striking contrast to the behavior of anionic dyes, such as eosin and rose bengal. The latter, according to Dognon (1927), are more potent photodynamically in acid than in alkaline solutions.

Independent action of buffers. The experiments on acidulated sea water showed that for ranges below a pH of 5.5 larval behavior depended only on the hydronium ion concentration, regardless of its source of maintenance, but at pH values higher than this the cations of some buffers had an independent action. Thus, phosphate and acetate buffers (both 0.05 *M*) as well as hydrochloric and citric acids (0.1 N) all gave identical results below a pH of 5.5. But within a range of 6.0 to 7.0 both sodium and potassium McIlvaine buffers caused the larvae to shed adhesive fluid while swimming, always as granular particles in sodium-buffered sea water but as reticulated fibers, which on congealing entangled the larvae, in potassium solutions. When the loss of adhesive fluid was great, metamorphosis did not occur. A slighter loss, while permitting metamorphosis, resulted in zooids that were abnormally long and slender, apparently lacking much of their zooecial walls, which are formed from adhesive fluid (*cf.* Corrêa, 1948). There is no adequate explanation for this peculiar loss of adhesive fluid in sodium- and potassium-buffered sea water. A similar phenomenon occurs in larvae subjected to heat (32°–35° C.), to potassium-free sea water, to tissue extract prepared from minced frog muscle according to the manner described by Harding (1951) and presumably containing a thrombin-like substance and, finally, to sea water raised to a pH of 8.8–9.5 by sodium or potassium hydroxides. The latter, of course, cause calcium and magnesium precipitation, but the larvae show no loss of fluid in either magnesium-free or calcium-free sea water.

TABLE III

The effects of acidity on the induction of metamorphosis by neutral red dye. The concentration of neutral red for both groups = 1:300,000 pts.

A. Number and percentage of metamorphosed larvae in sea water-dye at a pH of 6.8 at the end of one hour.				B. Number and percentage of metamorphosed larvae in sea water-dye at a pH of 7.8 at the end of one hour.		
No. larvae	No. metamorphosed	Per cent	Subst. added	No. larvae	No. metamorphosed	Per cent
74	47	64	HCl	55	23	42
90	10	11	HCl	64	27	42
35	12	34	Acetate	82	51	62
46	25	54	Acetate	72	40	54
15	2	13	Acetate	60	37	62
114	19	17	Acetate	68	42	62
133*	1	0.8	Acetate	130	85	65
507	116	22.8		431	305	70.7

The t ratio for the difference between larvae at a pH of 6.8 and at 7.8 = 2.4. $P = < .05$.

* This was the only stender dish covered. Note the effectiveness of covering the dish in preventing a rise of pH.

In contrast to the effects just discussed, neither acetate nor borate buffers (both 0.05 *M*, initial concentration) nor glycine (0.1 *M*, initially) had any noticeable effect on the larvae. Glycine and acetate buffers are of special interest, since Grave and Nicoll (1934) reported that the former had accelerating effects on ascidian metamorphosis; and the latter sometimes has an independent effect, as it apparently does when used on *Fundulus* eggs (Loeb, 1915; Bridges and Sumwalt, 1934). Statistical tests, made by using glycine in the small amounts employed in buffering sea water, did not reveal any significant effects of this amino acid. The possible effects of larger amounts of glycine are not yet known.

Discussion

Nothing need be said at this time regarding the photodynamic action of neutral red in the induction of metamorphosis. The effects of both light and dye on protoplasmic viscosity will be discussed in a subsequent paper concerning extrinsic factors in metamorphosis and parthenogenesis.

The anaesthetic effects of both alcohol and potassium cyanide lend support to the hypothesis that inhibition of metamorphosis may involve a decrease in protoplasmic viscosity. Thus, alcohol, potassium cyanide, low temperatures (5–12° C.), calcium-free sea water and an excess of potassium and magnesium ions all have two things in common; they inhibit metamorphosis in bryozoan larvae and, according to the calcium-release theory of Heilbrunn (1927, 1934, 1952, pp. 730–732), they either lower viscosity (potassium and magnesium ions, anaesthetics and low temperatures, the last in only some types of protoplasm at 5–15° C.) or they prevent a rise in viscosity of the interior of cells (calcium-free sea water) requisite for stimulation. Note should be made of the fact that potassium cyanide has effects other than anaesthesia alone; but it would be useless to discuss these in our present state of ignorance of respiratory enzymes of larvae. Unfortunately, the experiments with potassium cyanide do not yield any information concerning a possible relationship between mitotic inhibitors and those which suppress metamorphosis, since cleavage is stopped in some marine eggs by cyanide but not in others (*cf.* Brachet, 1950, p. 165).

Since the data on mechanical agitation indicate that shaking has no effect on metamorphosis other than that of preventing the larvae from acquiring the degree of quiescence requisite for setting, these results might seem to militate against the working hypothesis that the induction of the process of attachment is a kind of stimulation involving an increase in viscosity, possibly of a muscle or nerve of the organisms. Yet, mechanical agitation is by no means a universal method of effecting artificial parthenogenesis, presumably also a response to stimulation, since some kinds of marine eggs respond to this agent and others do not. Contrary to what one might expect, the forced activity of larvae during mechanical agitation does not cause an increased assimilation of neutral red from sea water by repeated collisions between the organisms and dye molecules. Neither does crowding have any effect on these organisms (unpublished data). Bryozoan larvae in these respects differ from ascidians, which assimilate more copper from sea water during mechanical agitation (or during crowding) than they do normally (Grave, 1935).

The effects of acids, however, are similar in both bryozoans and ascidians. Bradway (1936) found that setting of the tunicate, *Clavelina huntsmani*, was inhibited below a pH of 6.0 and that the percentage of attached organisms increased progressively as the pH rose from 6.0 to an optimum at 8.0; then a slight decrease occurred between 8.0 and 8.4. Bradway believed that a high pH favored the action of proteolytic enzymes which she thought were involved in setting. Grave (1935), working on other types of ascidians, obtained results essentially similar to those of Bradway. Berrill (1930, 1947), however, after removing larvae hatched at a high pH to a medium of low pH, concluded that the latter favored metamorphosis. But he also observed that tadpoles reared throughout at a high hydronium ion concentration became acclimatized; thus, absolute acidity was less effective than a sudden increase in the hydronium ion concentration. Unfortunately, Berrill's papers do not indicate what precautions he took to maintain the pH at a definite level, whereas Bradway kept a constant pH by changing the solutions every fifteen minutes. This controversy has never been settled.

There are many striking similarities in the effects of acidity on various organisms. Thus, acidity not only inhibits metamorphosis in bryozoans and in some, at least, of the ascidians, but it also causes a profound depression in the division rate of *Chilomonas* below a pH of 5.5 (Mast and Pace, 1938). It likewise inhibits cleavage in marine eggs. As is well known, cell division of the latter does not occur in acid solutions, even though these media may initiate mitosis after the organisms have been removed to normal sea water (Loeb, 1913; Lillie, 1926). Inhibition of fertilization and maturation by acids is also well known for many types of eggs (Smith and Clowes, 1924; Krahl, Clowes and Taylor, 1936; Tyler and Scheer, 1937; Hollingsworth, 1941; Allen, 1953). Furthermore, acidity inhibits the parthenogenetic effects of heat on *Nereis* eggs (LeFevre, 1945) and of ultraviolet light on *Spisula* eggs (Allen, 1953).

Finally, the motility of many organisms, other than bryozoan larvae, is greatly reduced in acid solutions. Marine amebae, for instance, show a progressive loss of movement as the pH drops from 8.5 to 5.9 (Pantin, 1923); and the fresh water species, *Amoeba proteus,* becomes immobilized in solutions on low pH, about 5.0 to 5.3, depending on the salt concentration (Pitts and Mast, 1933a; 1933b). Although one does not usually associate acid effects with anaesthesia, there are many similarities between the former and the latter. Not only do bryozoan larvae show identical behavior in acid solutions and in anaesthetics such as alcohol, potassium cyanide and an excess of magnesium or potassium ions, but other ciliated animals act as if anaesthetized in acidulated sea water (pH of 5.5), being apparently unable to transform chemical energy into the kinetic energy of motion according to Rogers (1938). (For anaesthetic effects of acid and alkaline solutions on *Nitella cf.* Osterhout and Hill, 1933.)

That bryozoan larvae would show a persistent retardation of the rate of metamorphosis on removal from acidulated or cyanized sea water was not anticipated. The problem of stimulation following inhibition, which occurs in a surprisingly large number of biological phenomena (*cf.* Buchanan, 1938), and the correlated question of the role of acidity in the application of the calcium-release theory to bryozoan metamorphosis will be presented in a subsequent paper. The above data lend support to the theory that a cationic dye, such as neutral red, induces setting on the alkaline side of the isoelectric point by releasing and supplanting calcium from its protein-binding. The freed calcium could then unite with protein molecules which act like anions above the isoelectric point of the protein involved. This hypothesis offers an explanation for the effects of cationic dyes in inducing both metamorphosis and parthenogenesis. (For the latter, *cf.* Brooks, 1947, 1949.) In acid media, however, calcium, even if freed by dye, could not unite with protein molecules which themselves act like cations in sufficiently acid solutions. Thus clotting could not occur. But both cationic dyes (neutral red and methylene blue) and at least one anionic dye (eosin) hasten the rate of fixation of *Bugula* larvae. (For the effects of anionic dyes in causing parthenogenesis, *cf*. Lillie and Hinrichs, 1923; Alsup, 1940, 1941.) At the pH of sea water, however, eosin is much less effective than neutral red in inducing metamorphosis. Since factors other than calcium are involved in coagulation, it would not be illogical to assume that anionic dyes cause stimulation by an alternative series of reactions involving other components of the clotting mechanism. But observations being made currently by the writer and one of his students show that both neutral red and eosin release calcium in *Elodea* leaves and cause the formation of oxalate crystals. The data so far available show that neutral red is more effective at a high pH, whereas eosin releases more calcium at a low pH. This is to be expected, if penetration is the main factor in causing a differential release of calcium at higher and lower pH values. Observations made by Beck (1933) on starfish eggs showed that neutral red penetrates more rapidly from media more alkaline than the protoplasm. Eosin, on the other hand, presumably acts like other acid dyes such as methyl red and fluorescein, both of which penetrate cells better at a pH lower than that of sea water. (*Cf*. Beck, 1933, for methyl red and Blum, 1941, p. 89, for fluorescein.) Undoubtedly much of the reduction in potency of neutral red in inducing metamorphosis in acid media is attributable to poorer penetration, for the larvae stain less intensely than at higher pH values. Nevertheless, other factors seem to be involved in the inhibition of neutral red by acidity, for some dye does penetrate the larval tissues. The problem of stimulation by dyes, and its relation to calcium-release, will be considered in a subsequent paper.

SUMMARY

1. Both absolute ethyl alcohol and 0.001 *M* KCN inhibit metamorphosis. A 3.3% solution of the former completely suppresses setting. But sea water containing 0.001 *M* KCN merely retards the onset of metamorphosis and reduces the number of attached forms in comparison to the controls. Inhibition by KCN persists for at least an hour after removal of the organisms to sea water. The induction of metamorphosis by neutral red is greatly inhibited by a 3% alcohol solution when the concentration of dye is low (1:300,000) but very little when the concentration of dye is high (1:100,000). Lowering the concentration of either factor while the other remains constant reduces the degree of antagonism.

2. Neutral red acts photodynamically in the induction of metamorphosis.

3. Mechanical agitation, for the duration of shaking used in these experiments, does not affect the rate of metamorphosis at the end of one hour or at the end of two hours after agitation ceases. But ten minutes after shaking the number of larvae metamorphosed in the control groups is almost significantly larger (P = .08) than that of the larvae exposed to agitation. This may indicate that shaking merely interferes with the mechanical process of setting, which requires quiescence on the part of the organisms, and does not affect the state of larval protoplasm.

4. When the pH of sea water is dropped to 5.5 the motility of the larvae decreases to nearly zero. Metamorphosis, also, is inhibited by a pH below 5.8 or 6.0. Acid inhibition of metamorphosis persists after the larvae are removed to their normal medium from sea water acidulated to the pH values used in these experiments (5.5 and 4.5). Below a pH of 5.5 the behavior of the larvae depends on the

hydronium ion concentration alone, regardless of the agent used to drop the pH of sea water. Within a range of 6.0 to 7.0, however, both sodium and potassium McIlvaine buffers interfere with setting and cause the larvae to lose adhesive fluid while swimming. At the concentrations employed in the experiments, no significant difference in the behavior of experimental and control larvae could be detected when glycine, borate or acetate buffers were added to sea water. The initiation of metamorphosis by neutral red is inhibited partially within a pH range of 6.5–7.0 and completely at a pH of 5.5.

5. The data support the hypothesis that inhibition of metamorphosis is actually a kind of anaesthesia brought about by a reduction in viscosity of the larvae or of some of their tissues such as muscles or nerves. These experiments and others can be interpreted best by assuming that the initiation of metamorphosis in bryozoans is a response to stimulation involving viscosity changes in the protoplasm opposite to those ensuing when inhibition occurs.

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IONIC AND OSMOTIC CONCENTRATIONS IN BLOOD AND URINE OF PACHYGRAPSUS CRASSIPES ACCLIMATED TO DIFFERENT SALINITIES

C. LADD PROSSER, JAMES W. GREEN AND TSAIHWA J. CHOW[1]

University of Illinois, Urbana, Illinois; Rutgers University, New Brunswick, New Jersey; University of Washington, Seattle, Washington

In its normal medium of sea water the blood of *Pachygrapsus* may be slightly hypo-osmotic (Jones, 1941; Robertson, 1953). In a dilute sea water the blood concentration declines somewhat but is maintained higher than the concentration of the medium (Jones, 1941) and this hyper-osmotic regulation permits the crab to enter regions of brackish water. In a more concentrated sea water *Pachygrapsus* shows limited hypo-osmotic regulation, a function associated with its survival out of water for long periods, as at low tide. Ionic analyses of blood as compared with sea water indicate that magnesium and sulfate are strongly excluded and that sodium, potassium and calcium concentrations are only slightly lower than in sea water (Robertson, 1953). If the crab is acclimated to sea water of various salinities, the ratio of potassium is held more constant than the sodium ratio (Gross, 1952).

The shore crabs' limited mechanisms of osmotic and ionic regulation are not well known but they are certainly interrelated. The excretory organs (antennary glands) appear to be more important for ionic than for osmotic regulation. In brackish water the osmotic concentration of the urine of *Carcinus maenas* is higher than the concentration of the medium and slightly lower than the concentration of the blood, yet the chloride in urine is the same as in blood; the volume of urine becomes increased but the combined effects of increased volume, slight dilution and no change in chloride concentration cannot account for the hyper-osmotic regulation (Nagel, 1934). A similar conclusion was reached on other grounds for *Palaemon* (Parry, 1954).

That various ions are excreted at separate rates is indicated by different blood/urine ratios for specific ions in such crabs as *Carcinus* (Robertson, 1949; Webb, 1940). Iodide which is injected into the crab is found to become concentrated in the urine (Nagel, 1934) and when extra $MgSO_4$ is added to sea water, magnesium and sulfate ions are increased in the urine (Webb, 1940) although urinary excretion accounts for loss of only a small fraction of injected salts (Bialasezwicz, 1932). To compensate for the high salt loss via urine when in a dilute medium, the crab may absorb salts actively, possibly by its gills (Webb, 1940); there may also be body salt stores which are used when the crab is in a dilute medium but these would necessarily be temporary (Hukada, 1932; Gross, 1951). The passive permeability of the body surface to both water and salts is low (Webb, 1940).

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Mechanisms of hypo-osmotic regulation in crabs are unknown, although active absorption of water is suggested (Gross, 1952). The mechanism of urine formation in the antennary glands is also unknown but filtration can hardly occur and secretion of salts and possibly of water is indicated (Prosser *et al.,* 1950).

A balance sheet of influx and outflux of water and ions by the various routes is needed. The following observations do not provide such a balance but they do partially indicate the role of the antennary glands in ionic and osmotic regulation in *Pachygrapsus crassipes*. *Pachygrapsus* is particularly suitable for investigation because it shows both hyper-osmotic and hypo-osmotic regulation, it can be catheterized easily for urine, it is of convenient size and survives well in the laboratory.

MATERIALS AND METHODS

Freshly collected specimens of *Pachygrapsus crassipes* were acclimated to 50% and to 170% sea water (S.W.) over periods of five to seven days by changes of 10 to 30% per day; preliminary experiments showed virtually complete adaptation to such salinity changes in 24 hours. The crabs were kept in approximately 100 ml. of fluid in individual finger bowls at room temperature and the water was changed once or twice daily. Records were kept of sexes, but no differences noted; however, marked abnormalities in osmoregulation were found in molting crabs, as observed by Baumberger and Olmsted (1928); hence soft-shelled crabs were eliminated.

Blood samples were removed from sinuses by means of glass capillaries of 1 mm. O.D. and 2 cm. length by puncturing the cuticle at a leg joint. Urine was sampled by inserting the tips of capillaries, 0.1–0.3 mm. diameter, into the excretory pore; normally the pressure in the bladder caused the urine to overflow a 10 cm. long capillary at once. Drops of blood and urine were extruded from the capillaries onto a paraffin surface for sampling by micropipettes. Osmotic concentrations were measured in both blood and serum, ions in serum only.

Osmotic concentrations were measured by a freezing-point method similar to that developed by Gross (1952). Capillaries containing a small amount (0.01 ml. is sufficient) of fluid are sealed at the ends with vaseline, mounted in grooved plastic racks and quickly frozen on dry ice. A number of these racks of capillaries can be accumulated and on each rack are placed four or five capillaries containing known concentrations of NaCl; each rack holds 12 capillaries. For measurement, a rack of frozen capillaries is inserted into a holder which is immersed in a 300-ml. dish of brine (2 N NaCl) cooled by dry ice to lower than − 2° C. A stirrer agitates the brine which warms at about 1° per 15 minutes, a rate fixed by the amount of insulation in the box supporting the dish. Light from below passes through a sheet of polaroid, then through the dish with the capillaries, and through a plastic cover on which is a second piece of polaroid. When viewed by polarized light, the transition from the crystalline frozen state to the melted state is abrupt and sharp. The time of complete melting in each capillary is recorded, this time is plotted against the known concentrations, and values of unknowns are obtained by interpolation and expressed as equivalent normality of NaCl. Results of this method are easily reproducible to within 0.02 *N;* addition of 0.5% albumin to sea water causes no significant difference and values for serum and clotted blood overlap.

Sodium and potassium concentrations were measured with a Beckman flame photometer, using an acetylene flame. For the urine, 10-μl samples were read directly after dilution with Pyrex-distilled H_2O; blood samples were allowed to clot and serum was drawn into the micropipette. Sodium was read at 588 mμ and K at 767 mμ, against appropriate standards. Magnesium and calcium concentrations were measured by a Beckman flame photometer equipped with a hydrogen flame and a multiplier phototube similar to that described by Chow and Thompson (1955). Calcium and magnesium were read at 422.7 and 370.8 mμ, respectively.

Seven separate series of experiments were used with variations as indicated under Results. The total number of crabs examined after equilibration were: 31 in 100% S.W., 32 in 170% S.W., 20 in 50% S.W.

RESULTS

In the first three series, osmo-concentration, potassium and sodium concentrations in blood (serum) and in urine were measured from groups of five or six crabs each in 50%, 100% and 170% S.W., respectively. In the fourth series some of the crabs from the second series were sampled a week later from 50%, 100% and 180% S.W.; these crabs were in poor condition, several deaths had occurred and it was concluded that experimental conditions were inadequate for such prolonged storage. In the fifth series of measurements, three groups of three crabs each were sampled at 1, 3 and 5 days after reaching 170% S.W. (this after 4 days of gradual transfer through intermediate concentrations); three groups of three crabs each in 100% S.W. sampled at the same time. No significant difference was found at the three times sampled; hence it was concluded that acclimation was virtually complete one day after reaching 170% S.W. Osmotic concentrations were measured on blood and serum in two-thirds of the animals, on blood in all. However, since differences between blood and serum values in a given medium do not differ by one standard error, only the blood figures are here presented.

The results of all the measurements of sodium, potassium and osmo-concentration in blood and urine of crabs acclimated to 50%, 100% and 170% S.W. are summarized in Table I and in Figure 1, b, c, and d and the significance in terms of 95 per cent fiducial limits for the blood are given in Table II. The osmotic concentration of the blood is clearly hypertonic to 50% S.W., it is insignificantly hypotonic in 100% S.W., and significantly hypotonic to 170% S.W. The urine is slightly hypotonic to the blood (10% level significance) in 50% S.W. and in 100% S.W. (3% significance). However in 170% S.W. the osmo-concentrations of blood and urine are virtually identical. It may be concluded that the antennary glands can play only a very minor part in hyper-osmotic regulation and no part at all in hypo-osmotic regulation.

The potassium concentration in blood from crabs in 100% S.W. is higher and in 50% S.W. is much higher than the potassium in the medium. The urine potassium concentration slightly exceeds that in the blood in crabs from 50% S.W. but this difference is not significant (10% level) and there is no difference in 100% S.W. and 170% S.W. It appears, therefore, that the antennary glands do not function in regulation of potassium but rather that urine potassium reflects blood levels.

Sodium in blood is more concentrated than in the medium when the animal is in 50% S.W. but the difference is less than for osmo-concentration; in other concentrations of sea water the blood sodium does not differ significantly from the medium. Hence osmotic regulation is determined mainly by other than

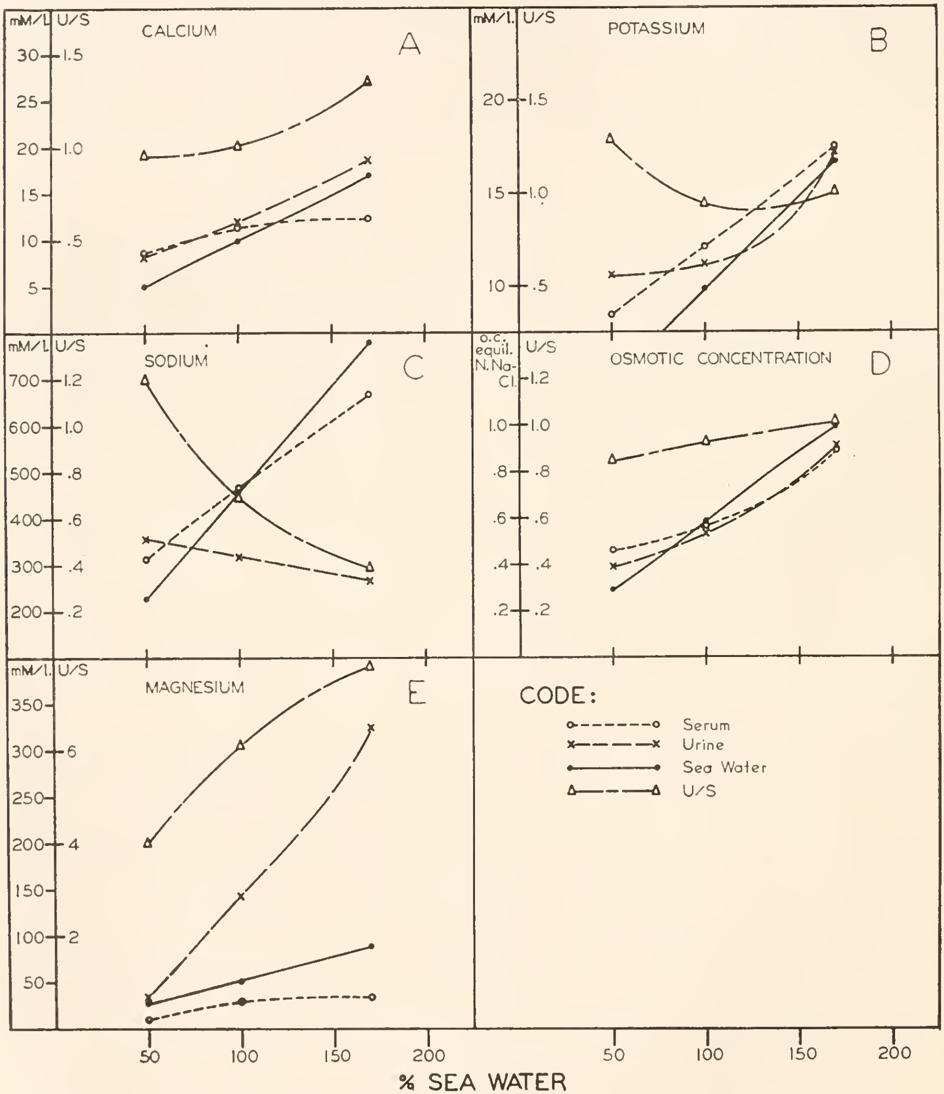

FIGURE 1. Average data for concentrations of calcium, potassium, sodium, serum, urine and external medium in m mols per liter and for osmotic concentration in equivalent normality of NaCl. U/S gives urine/serum ratios. Number of crabs tested in each concentration of sea water is given in Tables I and IV.

TABLE I

Sodium, potassium, and osmotic concentrations in blood and urine of crabs from 50%, 100%, and 170% S.W.

	50% S.W.			100% S.W.			170% S.W.		
	Avg.	S.E.	No. crabs	Avg.	S.E.	No. crabs	Avg.	S.E.	No. crabs
Sodium									
(mM/1) Serum	313	±12.0	18	465	±11.2	30	668	±12.0	31
(mM/1) Urine	356	±17.3	17	318	±16.7	27	264	±19.2	29
U/S ratios	1.2	± 0.1	17	0.7	± 0.04	26	0.39	± 0.024	29
Potassium									
(mM/1) Serum	8.4	± 0.78	13	12.1	± 0.65	30	17.4	± 0.66	31
(mM/1) Urine	10.6	± 0.94	13	11.2	± 0.59	28	17.2	± 0.77	31
U/S ratios	1.29	± 0.09	13	0.94	± 0.05	27	1.00	± 0.04	31
Osmotic concentration									
(equiv. N NaCl) Blood	0.46	± 0.026	18	0.57	± 0.013	24	0.89	± 0.023	30
(equiv. N NaCl) Urine	0.39	± 0.031	19	0.53	± 0.013	25	0.90	± 0.02	29
U/B ratios	0.85	± 0.04	18	0.93	± 0.03	24	1.00	± 0.01	29

sodium ions. However the sodium concentration in urine is higher than in serum (significant only at 5% level) in 50% S.W., is lower in urine than in blood when in 100% S.W. and much lower when in 170% S.W. Thus the sodium concentration in urine *decreases*, while in blood it *increases* with increasing environmental concentration.

TABLE II

Statistical significance of analyses

A. Test of concentrations in blood as compared with medium.

	50% S.W.		100% S.W.		170% S.W.	
	Blood 95% limits	S.W.	Blood 95% limits	S.W.	Blood 95% limits	S.W.
Osmotic conc. (equiv. N NaCl)	0.41–0.51	.29	.54–.60	.58	.84–.94	.99
Na (mM/1)	288–338	229	435–495	459	639–697	780
K (mM/1)	6.7–10.1	4.9	10.8–13.4	9.8	16.1–18.7	16.7

B. Test of blood and urine differences.

	50% S.W.		100% S.W.		170% S.W.	
	t	Significance	t	Significance	t	Significance
Osmotic conc.	1.68	$P < 0.1$	2.17	$P \sim .03$	.33	not sig.
Na	2.05	$P = .05$	7.3	$P < .01$	1.75	$P < .01$
K	1.80	$P > 0.1$	1.02	not sig.	.19	not sig.

TABLE III

Averages of analyses of blood and urine from crabs in artificial sea water (average of 3 crabs each)

	Na			K			Osmotic conc.		
	Serum	Urine	U/S	Serum	Urine	U/S	Blood	Urine	U/B
A. From normal artificial sea water.									
100% S.W.	455	319	.79	12.2	7.5	.61	.59	.60	1.06
170%	653	200	.31	14.0	9.8	.77	.78	.75	.96
B. From $MgSO_4$-free artificial S.W.									
100%	422	463	1.09	10.1	12.3	.95	.52	.53	1.01
170%	686	514	.75	13.5	15.1	1.11	.92	.93	1.01

Since urinary sodium decreases while urine osmo-concentration and blood osmo- and sodium concentrations increase, some other important solute must be replacing the sodium in the urine. Magnesium is the next most abundant cation in sea water. To test its possible role an experiment was conducted with crabs in artificial sea water, 100% and 170%, made up with and without $MgSO_4$. Sodium chloride was added to compensate osmotically in the $MgSO_4$-free sea water. Three crabs were acclimated in each concentration of each mixture. Results as given in Table III indicate that the sodium concentration in the urine decreased at the change from 100% to 170% artificial sea water as in normal sea water, but that in the absence of $MgSO_4$ the urine Na increased when the tonicity of the medium was increased. Also the urine sodium was higher in the $MgSO_4$-free 100% artificial S.W. than in the 100% normal S.W. It is concluded that the urine/serum ratio for sodium is reduced when Mg and SO_4 are present; that is, magnesium seems to suppress the urinary excretion of sodium.

In two series of experiments determinations of magnesium and calcium were also made. The data for Na, K and osmotic concentration in these animals have

TABLE IV

Summary of Mg and Ca analyses

		50% S.W.		100% S.W.		170% S.W.	
		mM/l S.E.	No.	mM/l S.E.	No.	mM/l S.E.	No.
Mg	serum	8.9 ±.73	3	29.2 ±.93	4	33.1 ±.59	4
	urine	32.1	2	143.6 ±21.2	5	324.6 ±.43	6
	U/S	4.0	2	6.1 ±.86	3	7.8 ±.47	4
	Sea water	26.0		52.0		88.5	
Ca	serum	8.6 ±.5	3	11.4 ±.60	4	12.3 ±1.1	4
	urine	8.4 ±.34	3	12.0 ±.56	6	18.6 ±2.2	6
	U/S	0.97±.02	3	1.01±.04	4	1.36±0.2	4
	Sea water	5.0		10.0		17.0	

been included in Table I, and the data for Mg and Ca are summarized in Table IV and Figure 1, a and e. The calcium level in both serum and urine increased as the total concentration of the medium increased. In fact, calcium appears to follow the pattern of potassium.

Serum magnesium increased proportionately more than any other constituent measured, nearly four-fold in going from 50% to 170% S.W. and the urine magnesium increased by nearly eight times. Thus as urine sodium decreases, urine magnesium increases.

Discussion

The preceding results confirm previous investigations in indicating differences in degree of regulation of different blood components. For a 50% dilution of sea water the per cent change in the measured components of blood in order of decreasing regulation is: osmotic concentration 19, calcium 25, potassium 31, sodium 33, magnesium 70. For a 70% increase in concentration of the medium the per cent change, also in order of decreasing regulation, is: calcium 7.9, magnesium 13, potassium 44, sodium 44 and osmotic concentration 56. The values for Mg and Ca are based on fewer measurements than the others but it is evident that each component is regulated to a different degree and in a different order for dilution than for concentration. Hyper-osmotic regulation is not the converse of hypo-osmotic regulation. These observations, in general, agree with and extend those of Jones (1941), Robertson (1953) and Gross (1952).

The role of the antennary glands is indicated by urine/serum ratios in Tables I and IV. Deviation of the U/S ratio from unity is a measure of effectiveness of the antennary glands in regulation. In 100% S.W. this deviation is significant to better than the 1% level for sodium and magnesium; it is of borderline significance for potassium and osmotic concentration and insignificant for calcium. In 50% S.W. the U/S ratio deviates from unity significantly for magnesium, sodium and potassium, questionably for osmotic concentration. In 170% S.W. only sodium and magnesium show U/S ratios significantly different from unity. It may be concluded that the antennary glands function in eliminating magnesium at all levels and that they tend to retain sodium and potassium in a dilute medium. In 170% S.W. the sodium concentration is similar in the medium, in the serum and in the urine, hence no active excretion might be demanded. However, since the blood is hypotonic to the medium and urinary magnesium is greatly increased, some influx of salts must occur and extra-renal excretion of sodium is likely. In hypo-osmotic marine teleosts magnesium and calcium are excreted renally, sodium and potassium extra-renally (Smith, 1930), whereas in *Pachygrapsus* urinary potassium increases along with magnesium and thus separates from the route taken by sodium.

Most unexpected is the decrease in urine sodium as the total urine concentration increases. The experiments with artificial sea water and the direct analyses indicate that the preferential route of Mg excretion is renal and that excretion of Mg interferes with the excretion of Na by the antennary glands. Thus *Pachygrapsus* differs from *Leander* (Parry, 1954) where urine magnesium increases more with total concentration than sodium but the sodium does not decrease. It would be of interest to learn whether the inverse relation of sodium and magnesium results from competitive interference in an enzyme system or from some other type of blocking of the sodium transport system by magnesium. Calculations indicate that magnesium salts are not totally adequate to account for the osmotic deficit in the urine as sodium decreases. For example, if it be assumed that each of the cations is excreted with chloride, the isotonic coefficients in the range of urine concentrations are 1.82 for NaCl, 1.9 for KCl and 2.6 for the divalent salts (from Internat. Crit. Tables). The computed total milliosmolar concentrations due to salts in urine are in 50% S.W. 766 compared with 704 measured, in 100% S.W. 997 compared with 955 measured, and in 170% S.W. 1398 compared with 1615 measured. If it is assumed that magnesium and calcium are excreted with sulphate, the isotonic coefficients of these salts are 1.2 and the calculated total osmotic concentrations due to the salts in the urine are 719, 789 and 927 milliosmoles in the three sea water concentrations. Hence some unknown solute must be present in quantity in the concentrated urines. In any case, complex secretory activity, both renal and extra-renal, is indicated for osmotic and ionic regulation in *Pachygrapsus*.

SUMMARY

1. When specimens of *Pachygrapsus crassipes* were acclimated to 50%, 100% and 170% sea water the average blood osmotic concentrations were equivalent to 0.46, 0.57 and 0.89 normal NaCl, as compared with the medium of 0.29, 0.58 and 0.99, respectively. Urine osmotic concentrations in the same series were equivalent to 0.39, 0.53 and 0.90 normal NaCl.

2. Thus the crabs are hyper-osmotic in a dilute medium and hypo-osmotic in a concentrated medium; the antennary glands may function slightly in hyper-osmotic regulation but not at all in hypo-osmotic regulation.

3. Serum sodium concentrations in 50, 100 and 170% S.W. were 313, 465 and 668 mM, urine sodium 356, 318 and 264 mM corresponding to environmental concentrations of 229, 459 and 780 mM. Thus as the blood sodium increases the urine sodium decreases.

4. The osmotic deficit in the urine is accounted for in part by magnesium which in 50, 100 and 170% S.W. was in blood 9, 29 and 33 mM, in urine 32, 144 and 345 mM while the medium was 26, 52 and 88 mM, respectively.

5. In artificial sea water urinary sodium decreased at high external salinity but in the absence of $MgSO_4$, the urinary sodium increased.

6. Active outward transport of magnesium by the antennary glands in some way reduces the excretion of sodium.

7. Potassium in blood is well regulated in dilute medium, less well in more concentrated sea water. Calcium in blood is more concentrated than in the dilute medium, less concentrated than in the higher salinity medium.

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THE EFFECT OF ROENTGEN RAYS ON THE COLLOIDAL PROPERTIES OF THE STARFISH EGG

PETER RIESER[1]

Zoological Laboratory, University of Pennsylvania, Philadelphia, Pa., and the Marine Biological Laboratory, Woods Hole, Mass.

In order to determine how protoplasm is affected by irradiation, ordinary methods of microscopic examination are insufficient. For marked changes in the protoplasmic colloid, changes which might even involve death of a cell, are usually not reflected in the microscopic appearance of dead, fixed, or even of unfixed cells. Similarly, cytochemical methods usually fail to give evidence of alterations in the colloidal organization of the cell. In order to understand how roëntgen rays affect living matter one must take into account the fact that protoplasm is a living colloid and determine how irradiation affects this colloid.

Wilson (1950) discovered viscosity changes in the irradiated *Arbacia* egg during mitosis. He was, however, unable to detect such effects in the unfertilized *Arbacia* and *Chaetopterus* egg. These negative results were confirmed (Rieser, unpublished data) even with much higher irradiation doses (200,000 r) than those used by Wilson. One might suppose that colloidal changes nevertheless do occur in the unfertilized egg following irradiation but that they do not become manifest until the egg is in its mitotic cycle or is physiologically active in some other way. This view is in agreement with the common belief that living matter is more highly radio-sensitive while undergoing some physiological change than when it is relatively inactive. With these views in mind experiments were performed, and are here reported, to elucidate the effects of roentgen rays on the unfertilized starfish egg.

MATERIALS AND METHODS

Eggs from the starfish *Asterias forbesii* were obtained by removing the ovaries from ripe females and placing them in finger bowls containing sea water. The loose eggs were strained through a double layer of cheesecloth in order to remove all debris and placed in sea water-containing stender dishes in a constant-temperature water bath at 23° C.

Egg suspensions were irradiated in plastic dishes immediately after obtaining the eggs. The characteristics of radiation were 180 k.v.; 25 m.a.; 6000 roentgens per minute; target distance 9.5 cm.; equivalent inherent filtration equal to 0.2 mm. of copper. Two opposed, parallel, self-rectifying tubes were used simultaneously.

Protoplasmic viscosity determinations were made with the centrifuge method. Only those eggs whose germinal vesicles had broken down were used. Eggs in

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[1] This investigation was aided by a grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

sea water were placed in glass centrifuge tubes containing a pycnotic cushion of 0.73 *M* sucrose which was separated from the egg suspension by an air space. An Emerson electric centrifuge was operated at top speed (14,200 r.p.m.), developing a force of 22,600 times gravity at this speed. In each experiment eggs were subjected to this centrifugal force for increasing periods of time, from 5 seconds upward. At each centrifugation time a different aliquot of the same egg suspension was tested. In each aliquot 50 eggs were examined and the percentage of cells with hyaline zones was noted. In some of the experiments the number of eggs with fat caps was determined. A study was also made to determine if roentgen radiation alters the colloidal properties of the nucleoplasm. Eggs in the germinal vesicle stage were used. Measurements of nuclear viscosity were made with the falling nucleolus method (Harding, 1949). Eggs were arranged on the stage of a horizontal microscope, and the time required for the nucleolus to fall through the entire diameter of the nucleus was measured. This was usually repeated ten times with each egg. The author wishes to thank Dr. Clifford V. Harding for suggesting the experiments on the nucleus and for his advice and criticism of this part of the work.

Results

The results of the effect of roentgen rays on the relative protoplasmic viscosity and on the appearance of fat caps in unfertilized starfish eggs are presented in Tables I and II. Six experiments were performed using 50,000 r (Table I) and five experiments with 100,000 r (Table II). In general a far greater percentage of unirradiated eggs possess hyaline zones after centrifugation than do the irradiated eggs. This holds true over the whole range of centrifugation times employed and is particularly noticeable below 60 seconds, with both irradiation doses (50,000 r, Table I and 100,000 r, Table II). Thus, x-rays markedly increase the protoplasmic viscosity of the unfertilized starfish egg. The effect, however, is no more pronounced with the higher dose of x-rays. Both tables also show that

Table I

The effect of roentgen rays (50,000 r) on protoplasmic viscosity and fat release in the starfish egg

Time of centrifugation, sec.	Controls, aver.% hyaline zones	Irradiated, aver. % hyaline zones	Controls, aver. % fat caps	Irradiated, aver. % fat caps
5	2	0		
10	1	0		
15	2	0		
20	3	0		
25	4	1		
30	2	1		
35	10	0		
60	17	13		
90	28	15	40	60
120	17	15	51	17
150	31	12	41	49
180	36	18	24	66

TABLE II

The effect of roentgen rays (100,000 r) on protoplasmic viscosity and fat release in the starfish egg

Time of centrifugation, sec.	Controls, aver.% hyaline zones	Irradiated, aver. % hyaline zones	Controls, aver. % fat caps	Irraidated, aver. % fat caps
5	3	0	15	15
10	7	1	16	14
15	9	1		
20	12	3		
25	12	1		
30	16	6	7	14
45	36	32	12	34
60	50	30	27	56
90	18	6	12	22
120	32	4	20	38

there is a somewhat greater number of eggs showing fat caps after centrifugation in the irradiated eggs than in the non-irradiated controls. More striking is the relatively far greater proportion of irradiated eggs with fat caps despite the higher protoplasmic viscosity of the latter (and consequently a decreased tendency toward centrifugal displacement of intracellular components in these eggs). The ability of x-rays to increase the percentage of eggs with fat caps therefore demonstrates an intracellular release of lipid materials. In addition to viscosity changes and fat release two further effects of irradiation were noted following centrifuga-

TABLE III

The effect of roentgen rays on time of fall of nucleolus and the thixotropy of the nuclear colloid

Dose. r	Time for initial fall, sec.	Aver. of times for subsequent falls, sec.	Ratio initial time to aver. of subsequent times
0	187	171	1.094
	210	187	1.123
	200	168	1.190
	210	181	1.160
	211	167	1.263
	212	178	1.191
	152	150	1.013
	150	145	1.035
	174	162	1.080
	184	178	1.033
50,000	160	111	1.441
	157	133	1.180
	166	142	1.169
100,000	240	148	1.623
	216	167	1.293
	170	185	0.919
	149	131	1.137
	210	132	1.599
200,000	160	144	1.111
	187	160	1.169

tion. In unirradiated eggs exposed to prolonged centrifugation there is always a number of cells that become destroyed. In such cells, the membrane separates from the cytoplasm, giving the appearance of a so-called "ghost" membrane similar to that of the mammalian erythrocyte. Irradiation tends to prevent this "ghost" formation. Thus, for example, using 50,000 r and centrifuging for 150 seconds, 8% of the unirradiated controls were ghosts, as compared with 0% ghosts in the irradiated eggs. Irradiation was found also to prolong the centrifugation time required to produce shape changes (*i.e.*, elongation) of the eggs. Table III contains the data pertaining to the effects of x-rays on the nuclear colloid. In the second column, the time of initial fall of the nucleolus through the germinal vesicle is a measure of viscosity (since the temperature was constant, viscosity is proportional to time). Since there was too much variability of the time of fall of the nucleolus among individual eggs, a comparison of the nuclear viscosity in the control and irradiated eggs cannot be made. The last column in Table III gives the ratio of the initial time of fall of the nucleolus to the average of subsequent falls and is therefore an index of thixotropy of the nuclear colloid. Application of the t-test to the means of these ratios reveals that there exists no significant difference in nuclear thixotropy between the control and irradiated eggs.

DISCUSSION

A fundamental question arises through the finding that x-rays alter the protoplasmic viscosity of the unfertilized *Asterias* egg but not that of the unfertilized *Arbacia* or *Chaetopterus* egg. How may one account for this difference in colloidal behavior? The answer may be found if one examines the modes of maturation in each case. The stage of maturation, and particularly the time course in which it is attained in the starfish egg, as compared with the other two cases, appears to be of decisive importance in determining the radiosensitivity of the protoplasmic colloid. The *Arbacia* egg, when shed into sea water, is fully matured, both polar bodies having been formed within the ovary. In the *Chaetopterus* egg, maturation begins upon its removal from the ovary into sea water, progresses to the metaphase of the first maturation division and ceases. This stage is reached within 15 minutes after the eggs are placed into sea water. In the *Asterias* egg, however, according to Tennent and Hogue (1906), the first maturation division is not completed until 70 minutes, and the second division not until after 105 minutes, after its removal from the ovary. Since the procedures of washing the eggs, preparing them for irradiation, and the irradiation itself generally took at least an hour, usually more, the starfish eggs actually were in the process of maturation during the x-ray treatment, while the eggs of the other two species were not. Thus, during the time the x-rays acted on the starfish egg, the latter was undergoing a physiological process, that of maturation. This is in accordance with the belief that protoplasm is more highly radiosensitive when in a state of activity. As in the *Arbacia* egg during mitotic division where irradiation prolongs the mitotic gelation, so does the starfish egg show an increased protoplasmic viscosity following its maturation divisions. The direction of the viscosity change in the irradiated starfish egg, that of increase, is the same as that observed by Wilson (1950) in the irradiated *Arbacia* egg during mitosis. In the latter case, the viscosity of the treated egg remains high for a period of about two or three times that of the controls. Wilson postulates that the effect of irradiation is an alteration or destruction of heparin within the egg, thus favoring prolonged gelation. He cites literature in support of this belief. His hypothesis might also be advanced in the case of the unfertilized, irradiated starfish egg.

The increase, especially seen as a relative increase, of fat caps following irradiation, is not surprising in view of previous studies where irradiation is known to increase the number and size of fat droplets in cells (Nadson and Stern, 1931), and in view also of the lipemia in mammalian blood following total-body irradiation. The effect may be due to a breakdown of protoplasmic protein-lipid complexes into proteins and lipids.

The tendency of irradiated starfish eggs to resist centrifugation-induced shape changes and membrane separation suggests that x-rays increase the cortical, or surface, rigidity of the eggs as they increase the viscosity of the interior protoplasm.

SUMMARY

1. Unfertilized eggs of the starfish, *Asterias forbesii,* were irradiated with single x-ray doses of 50,000 r and 100,000 r, respectively.

2. Roentgen irradiation produces a marked increase in protoplasmic viscosity. An explanation is given for the absence of colloidal changes following irradiation of eggs of two other species.

3. Two further radiation effects are: intracellular release of fat, and increase in cortical, or surface, rigidity of the starfish eggs.

4. Doses as high as 200,000 r fail to have a detectable influence on the nuclear colloid of the starfish egg.

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THE RESPIRATION OF SPISULA EGGS[1]

EVELYN SCLUFER[2]

Department of Zoology, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Massachusetts

Warburg, in 1908, found an increase in oxygen consumption after fertilization in the sea urchin egg. This seemed to support Loeb's theory (1906) of increased oxidative activities in connection with fertilization and development. Other workers subsequently found that eggs from other invertebrate animal groups showed no change at fertilization or even a decrease. It was eventually postulated that the change in oxygen consumption might be correlated with the stage of nuclear maturation at which fertilization occurs.

Whitaker (1933) removed the emphasis from oxidation as a determining mechanism in fertilization and, instead, posed the problem as one of understanding the differences in the metabolism of unfertilized and fertilized eggs. He found that unfertilized eggs of different species consume oxygen at very different rates, but that fertilization brings these rates closer together. Fertilization, therefore, seems to regulate the metabolism of the unfertilized egg to a level characterized by the more uniform needs of the developing organism.

Recently, Borei (1949) found that the change in oxygen consumption of sea urchin eggs at fertilization depends on the time after shedding at which fertilization occurs. The rate of the unfertilized egg gradually decreases, but the rate of the just-fertilized egg attains the same value regardless of the time elapsed between shedding and fertilization.

Biochemical studies on the nature and activity of the respiratory enzymes were conducted by many investigators, and especially by Runnström and his group. Runnström (1928a, 1928b, 1930) hypothesizes that colloidal rearrangements in the protoplasm, which are brought about by fertilization, change the spatial arrangement of the enzymes causing a change in the respiratory rate. Many protoplasmic changes in the egg have been observed in connection with fertilization (Runnström, 1949). Heilbrunn (1915, 1917, 1952) also connected changes in viscosity and other properties of the protoplasm, brought about by the calcium ion, to the events at fertilization.

Preliminary experiments with the egg of the surf clam, *Spisula solidissima* (Dillwyn), indicated that a decrease in oxygen consumption occurs after fertilization. It was thought that an examination of eggs from another animal group might help to determine which of the established respiratory properties of the sea urchin egg can be considered to represent general properties of an egg cell at fertilization, and which are merely special adaptations of the sea urchin group. Further measurements were made during the early development of the embryo.

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Materials and Methods

The surf clam, *Spisula solidissima* (Dillwyn), was obtained at the Marine Biological Laboratory at Woods Hole. The spawning season starts in the early part of June and lasts until late September. Fertilization of the eggs takes place externally and before the meiotic divisions have started. The first four cleavages are well synchronized and the embryos are swimming blastulae after 5½ hours at 21° C.

Eggs and sperm were obtained and handled according to the methods described by Allen (1951). Testes can be stored at 5° C. up to three days without impairing the fertilizing ability of the sperms. Washed eggs kept at 18° C. can be fertilized up to ten hours after removal from the ovary.

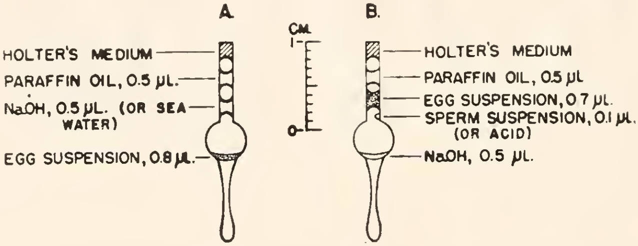

Figure 1. Cartesian diver charges. A. Loaded for O_2 uptake (or CO_2 output) measurements. B. Loaded for measurements during the course of which sperms or other substances are added.

The Cartesian diver respirometer was used in order to minimize crowding, to avoid damaging the eggs by shaking and to keep the cell number low in order to facilitate accurate cell counts. For measuring techniques see Linderström-Lang (1943), Holter (1943) and Borei (1948).

An egg suspension of 0.8 μl. was placed in the bulb of the diver. The first neck seal contained 0.5 μl. isotonic (0.47 M) NaOH, the second 0.5 μl. paraffin oil (Fig. 1). The control diver contained only sea water in addition to the seals. In order to facilitate loading and measurements and to avoid mechanical damage to the eggs by surface tension phenomena, the interior of the diver was coated with a hydrophobic layer of silicone. Either phenylsilicone (9989–1) or Dri-film (9987), manufactured by the General Electric Company, was used (Schwartz, 1949).

After measurements were completed, the eggs were removed from the respirometer and examined. Unfertilized eggs were tested for their fertilizability. The development of fertilized eggs was compared with that of control eggs kept in dishes in the respirometer water bath. Only those experiments were accepted in which unfertilized eggs showed over 95% fertilizability or fertilized eggs over 95% normal development.

The rate of development increases linearly between 18–23° C. At higher temperatures the rate of increase falls off. In order to secure a high respiratory rate, but at the same time not to operate too near the range of abnormal development, 21° C. was chosen for the experiments. The rate of development was determined by counting the number of nuclei and following their increase in number with time. Sections were made in order to study other embryological changes occurring.

For nuclear counts, the method described by Zeuthen (1951) for *Ciona* eggs was found much superior to whole mounts of fixed and Feulgen-stained eggs. Zeuthen's method consists of a careful and gradual compression of the eggs under a cover slip by withdrawing the suspension medium with tissue. The compression prevents further cleavage. Under the phase contrast microscope, the nuclei appear to be bright blue and are easily counted.

Sectioned material (10 μ) was obtained from eggs fixed in Zenker-formol, stained with toluidine blue and imbedded in paraffin.

Results

Morphological changes during development

The morphology of the shed *Spisula* egg and its changes upon fertilization have been described by Allen (1953), who followed the events up to 100 minutes after fertilization. The present study corroborates his findings and extends the ob-

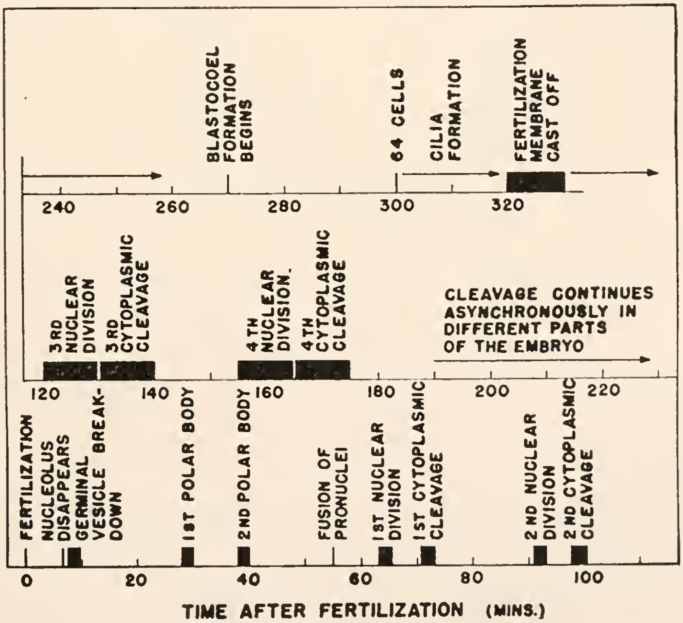

FIGURE 2. Development at 21° C. of *Spisula* eggs up to 330 minutes after fertilization. The width of the bands indicates the variation in time found for some of the respective events. The center of the band coincides with the time at which 50% of the embryos have completed the denoted event.

servations to 330 minutes after fertilization. Both studies were made at 21° C. The entire sequence is presented in Figures 2 and 3, in which the morphological events are related to time after fertilization. These eggs develop very synchronously up to the end of the second cleavage. It takes only two to three minutes for all of the embryos to complete a particular phase during this period. Thereafter, the synchrony between the individual cells in an embryo is less strict, but the embryos as a group continue to behave very synchronously.

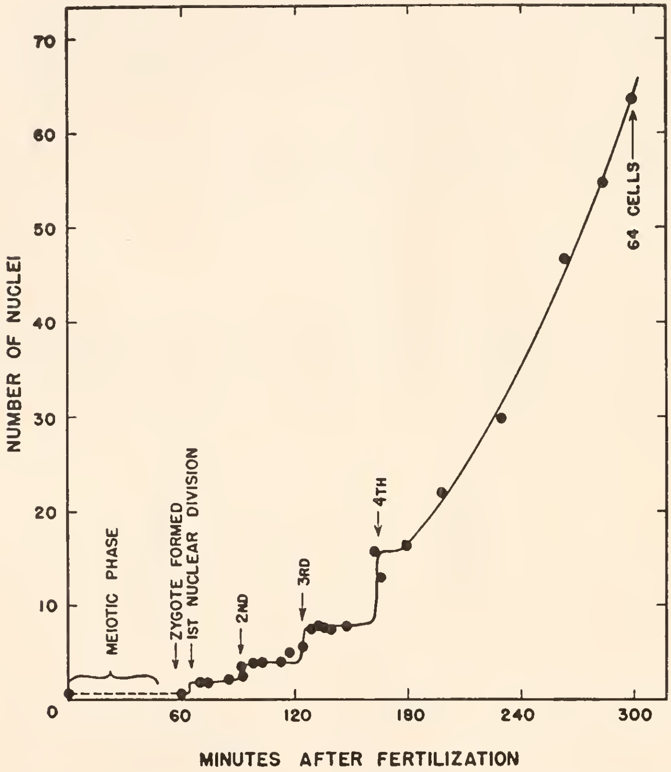

FIGURE 3. The increase in the number of nuclei during the early development of *Spisula* at 21° C.

Oxygen uptake

The oxygen consumption rate of 34 batches of unfertilized eggs was measured for periods up to eight hours after removal of the eggs from the ovary. Average values for ½-hour intervals are given in Figure 4. If the curve, which is drawn according to the method of least squares, is interpolated back to zero time, the eggs have a mean rate of 3.8×10^{-5} μl. $O_2 \times \text{egg}^{-1} \times \text{hour}^{-1}$ immediately upon shedding. After eight hours, the rate has dropped to 3.0×10^{-5} μl. and the Student's t-test shows the drop to be highly significant ($P < .001$). The deviations in the earlier part of the curve are probably associated with differences in time elapsed between the removal of the eggs from the ovary and the loading of the diver. This time varied from 0.5–2.5 hours.

The oxygen consumption during early development up to the swimming blastula stage was measured on 25 batches of eggs. Difficulties were encountered in measuring the rate just after fertilization. Unsuccessful attempts were made to fertilize the eggs inside the diver. According to Metz and Donovan (1949), the fertilizin secretion of these eggs is of a very high order. Fertilizin is known to inhibit fertilization and, since the eggs for technical reasons have to rest in the diver for times up to one hour prior to fertilization, its secretion may thus have prevented the fertilization process. The fertilization, therefore, had to be performed before the eggs were loaded into the diver.

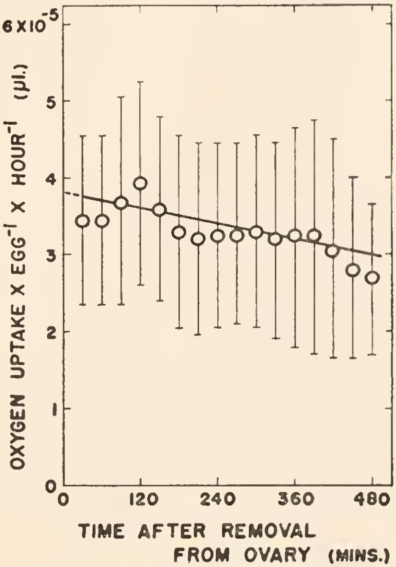

Figure 4. Rate of oxygen uptake at 21° C. of unfertilized *Spisula* eggs. Average values from 34 experiments. σ-values given for scattering.

Figure 5 shows the average values for ½-hour intervals after fertilization. Extrapolating back to ½-hour after fertilization gives a value of 3.0×10^{-5} μl. $O_2 \times$ egg$^{-1} \times$ hour^{-1}. A gradual increase is shown throughout the early cleavage stages up to the swimming blastula stage. The deviations in the early part of the curve may be due, as in the case of the unfertilized eggs, to the technical prehistory of the eggs and consequently to the different times at which measurements were begun. In most cases, loading was carried out immediately after fertilization and measurements begun after about ½-hour of equilibration. In some cases, however, loading was carried out after the first or second cleavage had taken place. The scattering recorded in Figure 5 is for the most part due to differences in rate between individual batches of eggs (biological scattering), rather than to spread between the points in each individual series of measurements.

The time at which eggs are fertilized after their removal from the ovary does not affect the post-fertilization rate. The rates at 60 minutes after fertilization were plotted against the time after removal at which fertilization was performed, and a straight line was fitted to the values using the method of least squares. The t-test showed the slope of the line to be insignificant ($0.40 < P < 0.45$).

Carbon dioxide output and respiratory quotient

The average of 11 experiments on the carbon dioxide output of unfertilized eggs gives the value 2.41×10^{-5} μl. $CO_2 \times egg^{-1} \times hour^{-1}$ for the time 200 minutes

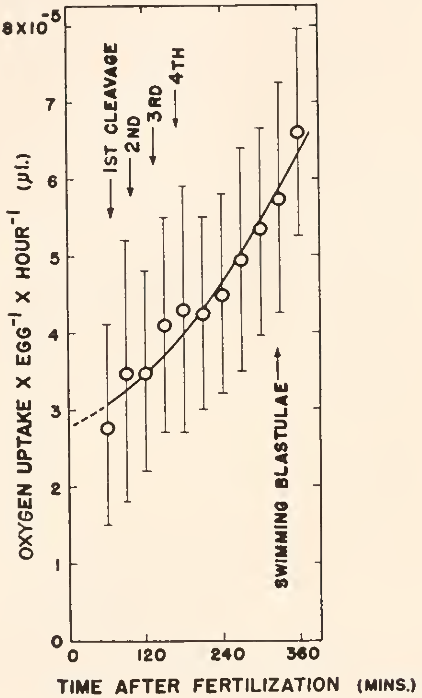

FIGURE 5. Rate of oxygen uptake at 21° C. of fertilized *Spisula* eggs. Average values from 25 experiments. σ-values given for scattering.

after removal from the ovary. The σ-value is 0.42. The R.Q. before fertilization is thus apparently much less than unity, probably in the vicinity of 0.7. It must be kept in mind that CO_2 measurements with the diver technique are inherently less accurate than O_2 uptake measurements. The reasons for this are two-fold: 1) leakage of CO_2 from the inside of the diver through the neck seals into the outside medium, and 2) errors introduced by the CO_2 retention properties of the eggs and sea water. Leakage through the seals was minimized in the experiments by use of an acid oil seal. Experiments on the retention properties indicate that the amount of CO_2 bound was not substantially changed during the time course of an experiment.

Nine runs on the CO_2 output after fertilization indicated that there is no perceptible change in the R.Q. value immediately after fertilization, but that later on a gradual increase toward unity seems to occur. The measurements comprise the time between fertilization and the swimming blastula stage.

DISCUSSION

A decrease in oxygen consumption rate of unfertilized eggs with time after removal from the ovary has been found by Holter and Zeuthen (1944) in *Ciona,* Zeuthen (1946) in *Rana* and Borei (1948) in *Psammechinus* and *Asterias.* In these cases, a constant rate was reached two to three hours after removal. In *Spisula,* the oxygen consumption rate of the unfertilized egg continues to decrease linearly for as long as eight hours after removal from the ovary. All of the above cited measurements were carried out with the Cartesian diver. No decrease in rate was found by Cleland (1950) in *Ostrea commercialis,* the rock oyster. Cleland employed the Warburg technique. It may be that the differences in technique could account for the differences in observations. However, unpublished data of Borei on sea urchins and of the author on *Spisula* indicate that Warburg and Cartesian diver measurements give similar results.

Cleland (1950) found no effect of fertilization on the oxygen consumption rate of *Ostrea commercialis.* Ballentine (1940), however, found a rise in respiratory rate at fertilization in the egg of *Ostrea virginica.* A large increase was also found by Ballentine (1940) in *Mactra lateralis.* In neither case did the latter author account for the time after removal of the eggs from the ovary at which fertilization took place. It is reasonable to assume, however, that one to four hours may have elapsed. The ratio in *Mactra* of the rates of fertilized eggs to unfertilized eggs is 1.8. Such a large value must indicate an increase in rate at fertilization over a long time span after shedding. *Mactra* and *Spisula* are very similar forms and have in common, among other things, the fact that the eggs are shed and fertilized in the germinal vesicle stage. In the oyster, however, the eggs proceed to the first maturation metaphase before they are fertilized. In the case of *Mactra* there is undoubtedly a rise in the respiratory rate at fertilization; in *Spisula,* there is a drop in rate at fertilization up to eight hours after shedding. The ratio obtained by Ballentine (1940) in *Ostrea virginicia* of 1.4 also indicates an increase in rate at fertilization.

Various workers (Shapiro, 1941; Tyler and Humason, 1937) have reported inconsistent results as to the direction and magnitude of the change in rate at fertilization in eggs of the same species and have ascribed this inconsistency to differences in individual batches of eggs. It is possible, however, that some of these differences could have been explained if the time of fertilization after removal of the eggs from the ovary had been taken into account. Recently Cleland (1950) tried to relate these differences to the kinetic state of the egg nucleus at the time of fertilization. This does not account, however, for the different behavior found in the two oyster species, *Ostrea virginica* (Ballentine, 1940) and *Ostrea commercialis* (Cleland, 1950) and in the two closely related molluscs, *Mactra lateralis* (Ballentine, 1940) and *Spisula solidissima.* The stage of meiosis at which the egg is normally fertilizable is probably not as important as the fact that both germinal vesicle breakdown and sperm entrance induce colloidal changes in the egg. (*Cf.* review by Runnström, 1949.) It is well known that colloidal changes have a paramount importance for the activity of the cytochrome system (Keilin and Hartree, 1949; Borei, 1950, 1951).

The independence of post-fertilization respiration from the level before fertilization which was found by Borei (1949) to exist in *Psammechinus* was found to be the case in *Spisula* also. The mean rate at thirty minutes after fertilization is 3.0×10^{-5} μl. $O_2 \times \text{egg}^{-1} \times \text{hour}^{-1}$. It was previously shown that the mean rate of the unfertilized egg varies from 3.8×10^{-5} μl. $O_2 \times \text{egg}^{-1} \times \text{hour}^{-1}$ upon shedding to 3.0×10^{-5} μl. O_2 eight hours later. If the eggs are fertilized immediately after shedding or soon thereafter, there is a drop in the oxygen consumption rate within thirty minutes after fertilization. The longer the time interval between shedding and fertilization, the less is the drop in rate. After eight hours, there is no change in rate at fertilization. It might thus be that if the eggs stood for even longer periods of time than eight hours before being fertilized, there might be an increase in rate at fertilization. This range of possibilities was encountered in other material by Tyler and Humason (1937) as mentioned previously.

The rate of oxygen consumption of *Spisula* eggs during development follows the general pattern of other forms which have been studied. A gradual increase is observed (Fig. 5).

The carbon dioxide output measurements indicate certain conclusions as to changes in R.Q. during the development of the egg. These conclusions are in accordance with previous findings on echinoderm eggs and others. (For a review see Brachet, 1950.) Recently, Borei (unpublished) found in *Lytechinus pictus* that the R.Q. immediately after fertilization has a value of 0.7 or even lower, but that during the course of early development a gradual increase toward unity takes place. The findings in *Spisula* point in the same direction. It might thus be permissible to conclude that the egg immediately after fertilization draws on fat reserves, but gradually switches to carbohydrates later on. Analyses of the fat components of the embryo during different stages (Hayes, 1938; Hutchens, 1942; Öhman, 1944) also bear this out.

A comparison of the sea urchin and the clam egg respirations might permit the following general conclusions to be drawn:

1. The pre-fertilization respiration of a marine egg is a quantity determined by internal substrate conditions, and subject to a variation in magnitude dependent on the time elapsed from shedding.

2. On fertilization, however, more defined species-specific rates are predominant, *i.e.,* rates connected with the morphogenetic work that has to be done by the developing embryo.

3. The pre- and post-fertilization respirations seem to be independent processes. The prevailing rate of respiration before fertilization has no influence on the rate attained after fertilization.

4. The R.Q. results seem to validate the conclusion that different sources of substrate are utilized before and after fertilization. These conclusions may be considered to agree with a general concept that the egg before fertilization has to maintain only a physiological state making it ready and suitable for fertilization, whereas after fertilization new mechanisms come into play which have the sole purpose to provide for the morphological and functional development and differentiation.

I wish to express my appreciation to Dr. Hans Borei for his help in planning this problem and for his helpful advice and criticism during the course of the investigation.

SUMMARY

1. The early development of *Spisula solidissima* (Dillwyn) up to the swimming blastula stage is described.

2. The oxygen consumption of the unfertilized egg decreases gradually after removal from the ovary. The mean rate immediately after shedding is 3.8×10^{-5} $\mu l.\ O_2 \times egg^{-1} \times hour^{-1}$; eight hours later the value is 3.0×10^{-5}.

3. There is a decrease in oxygen consumption rate within 30 minutes after fertilization. The magnitude of this decrease depends on the time which has elapsed between the eggs' removal from the ovary and fertilization; the shorter the time, the larger the drop.

4. The post-fertilization oxygen consumption rate is independent of the rate before fertilization. Thirty minutes after fertilization, it has a value of 3.0×10^{-5} $\mu l.\ O_2 \times egg^{-1} \times hour^{-1}$.

5. The oxygen consumption rate of the fertilized egg increases gradually during development.

6. Carbon dioxide measurements indicate a respiratory quotient of 0.7 before fertilization. After fertilization, there is a gradual increase toward unity.

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AN EXPERIMENTAL ANALYSIS OF THE DISCONTINUOUS RESPIRATION OF THE CECROPIA SILKWORM[1]

HOWARD A. SCHNEIDERMAN AND CARROLL M. WILLIAMS

Department of Zoology, Cornell University, Ithaca, N. Y., and the Biological Laboratories, Harvard University, Cambridge 38, Mass.

The breathing of insects has been an object of recurrent interest since the seventeenth century when Malpighi (1669) published his celebrated description of the tracheal system of *Bombyx mori* and correctly ascribed to it the function of respiration. Though other early investigators, including Lyonet, Réaumur, Bonnet, and Spallanzani, recognized that the spiracles were the sites where air made entrance into the tracheal tubes, they believed that expiration occurred elsewhere, through the mouth, or anus, or invisible pores in the skin (*cf.* Wigglesworth, 1931). It was Treviranus (1816–20) who first suggested that the spiracles provide the pathway for both inspiration and expiration—a view later amplified by the British physician, George Newport (1836). Newport was especially concerned with the respiration of lepidopterous pupae. Though apparently aware that breathing motions are indetectable in such pupae, he nevertheless supposed that the ventilation of the tracheae was due to active respiratory movements in which "nearly all the muscles and nerves of each segment are brought into consentaneous action as the muscles of the chest and ribs in vertebrated animals" (page 546).

Thomas Graham in 1833, having just discovered his law of gaseous diffusion, expressed a view contrary to that of Newport and suggested that simple physical diffusion of gases through the tracheal system might suffice for the respiration of most insects. Nearly one hundred years elapsed before experimental proof of this hypothesis was forthcoming. Then in a series of ingenious experiments of a type for which he was justly famous, Krogh (1920) showed that simple diffusion of oxygen and carbon dioxide through the spiracles and tracheae could more than suffice for the respiration of insects lacking respiratory movements. The essential elements of Krogh's theory have stood the test of time and have been most recently examined in detail by Thorpe and Crisp (1947).

On the basis of this long evolution of present thought concerning the mechanism of insect respiration, there was no theoretical basis for anticipating a series of puzzling observations which we first encountered five years ago in measurements of the respiratory quotients of diapausing pupae of the Cecropia silkworm. The R.Q. in these studies was found to undergo large and recurrent fluctuations as a function of time, in that prolonged periods of very low quotients were punctuated by more transient periods of very high quotients. It soon became evident that the source of the variability was a discontinuous release of carbon dioxide from the insects' tracheal system—a phenomenon which, we subsequently learned, had

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already been described by Punt in 1944 and attributed to a corresponding rhythm in the opening of the spiracular valves.

In the absence of measurements of oxygen uptake, Punt had apparently presumed that the uptake of oxygen, like the output of carbon dioxide, was discontinuous and synchronized with the opening of the spiracular valves. Consequently, Punt's work failed to reveal the true proportions of the respiratory paradox which became apparent only when the intake of oxygen was found to be *continuous* (Schneiderman, 1953; Schneiderman and Williams, 1953a, 1953b). This implies that during the period which separates the carbon dioxide "bursts," the spiracles permit the steady entry of oxygen while opposing the steady exit of carbon dioxide —a finding which appeared to be inconsistent with any simple theory of the diffusional transport of respiratory gases *via* the spiracle. Consequently, the discontinuous release of carbon dioxide has been studied in detail during the past five years. The present report considers a series of experiments which clarify the nature of the discontinuous respiration and provide a factual basis for theoretical interpretation.

MATERIALS AND METHODS

1. *Experimental animals*

The present investigation is based on approximately 1000 respiratory measurements performed on a total of 300 larvae, pupae, and developing adults of the giant silkworm, *Platysamia cecropia.* In occasional experiments pupae of other closely related saturniids were employed; namely, *Samia walkeri, Antheraea mylitta, Telea polyphemous, and Actias luna.*

2. *Manometric methods*

The respiratory exchange in most experiments was determined manometrically according to techniques previously described (Schneiderman and Williams, 1953a). Measurements were performed by the "direct manometric method" (Umbreit *et al.,* 1949) in 45-cc. cylindrical vessels equipped with venting plugs and adaptors for use with standard Warburg manometers. In studies of oxygen uptake the carbon dioxide output was absorbed on two corrugated strips of filter paper, moistened with a total of 0.3 to 1 cc. of 1 N NaOH or KOH and placed above and below the animal. Determinations were performed over periods that averaged about 8 hours, the excursion of the manometer being recorded at intervals of from 5 minutes to several hours as dictated by the rate of oxygen uptake. For the purpose of measuring carbon dioxide output, the filter paper was moistened with 0.3 to 1 cc. of 0.01 N HCl; manometer readings were then recorded over periods that averaged about 24 hours at intervals of from 2 to 30 minutes depending on the rate of carbon dioxide output. In experiments of long duration, the vessels were periodically removed from the bath, opened, and equilibrated with air for a few moments, to insure a constant gaseous environment. Although the metabolic rate of individual diapausing pupae is substantially constant over periods of at least a week, measurements of oxygen uptake and carbon dioxide output were ordinarily made within 12 hours of one another.

In one series of experiments, the gas exchange was measured volumetrically in a capillary respirometer. This and other special methods will be discussed in the appropriate sections. Each type of experiment, unless otherwise noted, was performed on a minimum of five animals.

The Cycle of Carbon Dioxide Release

Figure 1 illustrates a typical experiment descriptive of the cyclic release of carbon dioxide. Here the respiratory exchange of a Cecropia pupa was measured over two successive 18-hour intervals. During the first period of 18 hours, sodium hydroxide was placed in the vessel to absorb the carbon dioxide and thereby permit measurements of oxygen consumption; the alkali was then replaced by acid and the measurements repeated. As recorded in Figure 1, in the presence of alkali the gas volume of the respirometer decreased steadily during the entire 18-hour period, save for occasional extremely minor variations. But when acid was present to prevent the absorption of carbon dioxide, the gas volume exhibited

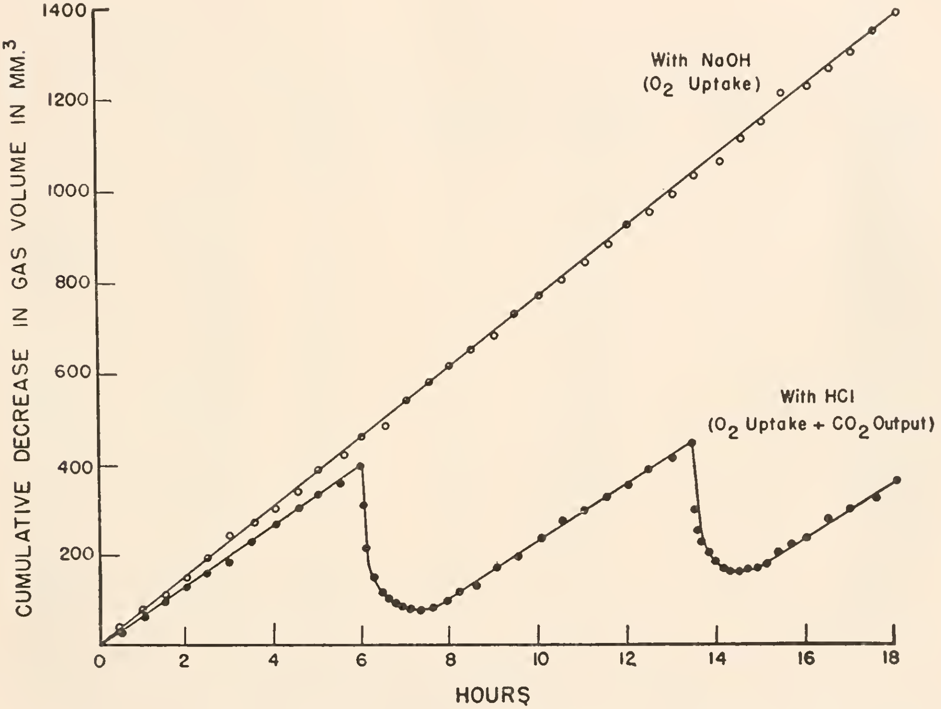


Figure 1. The gas exchange of a Cecropia pupa over two successive 18-hour intervals. The upper curve (open circles) records measurements performed in the presence of alkali; for the lower curve (solid circles) the alkali was replaced by acid. An apparent decrease in the rate of oxygen uptake followed by a slight increase was usually recorded at intervals which corresponded with the time of the carbon dioxide bursts. This result is an artifact due to inability of the alkali to absorb the large burst of CO_2 as rapidly as it evolved. By employing a larger filter paper surface for CO_2 absorption, this artifact could be almost completely eliminated.

tremendous discontinuities. In the experiment plotted, a steady decrease in volume is evident until the sixth hour. There then occurs an abrupt increase in volume which evidently signals a rapid evolution of gas from the insect.

Results of this type permit one to construct an over-all picture of the kinetics of oxygen uptake and carbon dioxide output, as shown in Figure 2. Here we see that the rate of carbon dioxide output over the 18-hour period was discontinuous, while the rate of oxygen uptake was apparently continuous. At approximately 6-hour intervals the animal gave off a burst of carbon dioxide; meanwhile, between successive bursts only 5 per cent of the total carbon dioxide was released. The carbon dioxide output during the first 12 minutes of the burst was 280 times larger than during a corresponding period between bursts. In this particular pupa, which weighed 5 grams and displaced about 5 cc., the average burst volume was 500 mm^3; *i.e.,* a volume of carbon dioxide far exceeding the total volume of the insect's tracheal system (see Discussion).

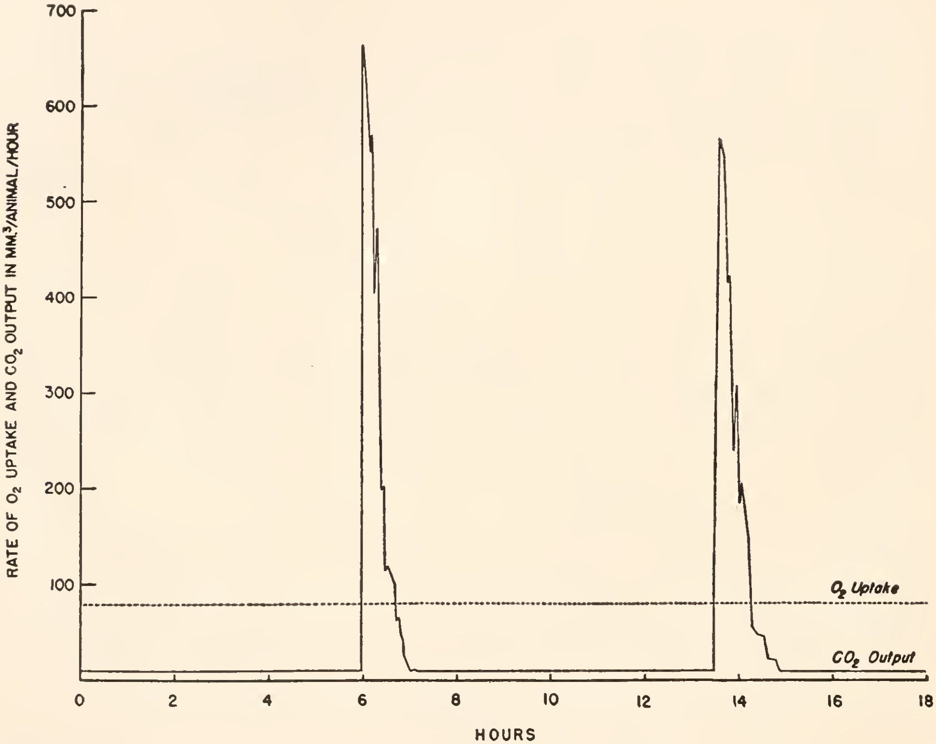


FIGURE 2. Rates of oxygen consumption and carbon dioxide output of a diapausing pupa over an 18-hour interval. The curve describing the carbon dioxide output was calculated from the data presented in Figure 1 by the "direct method" of Warburg.

Under constant experimental conditions, each individual repeats the recurrent cycle of carbon dioxide release day after day with only minor variations. But, among different individuals, considerable variations are encountered in the magnitude and timing of the cycles. For any particular animal the quantitative aspects of the cycle can be resolved into four parameters: (1) the average rate of oxygen consumption or carbon dioxide output over the whole cycle (*i.e.,* the metabolic rate); (2) the rate of continuous carbon dioxide release between bursts; (3) the duration of the cycle as measured from the end of one burst to the end of the succeeding burst (or its reciprocal, the frequency of bursts); and (4) the burst volume.

Table I records all of these parameters for a representative sample of the several hundred pupae on which respiratory measurements were performed. The values for each individual represent the average of at least two cycles. In the case of four individuals, duplicate determinations obtained after intervals of about a week have been included to illustrate the regularity and persistence of the cycles.

TABLE I

The cycle of carbon dioxide release of 14 normal diapausing pupae at 25° C.

Animal no.	Weight (grams)	Over-all rate of O_2 consumption (mm^3/gm. live wt./hr.)	Over-all rate of CO_2 output (mm^3/gm. live wt./hr.)	Interburst rate of CO_2 output (mm^3/gm. live wt./hr.)	Average cycle length (hours)	Average burst volume/animal (mm^3)
578	4.24	7.8	5.8	0.03	9.7	242
675	6.62	8.9	7.6	0	6.5	309
558(1)	4.55	9.2	8.3	2.4	14.3	386
558(2)	4.74	9.3	7.8	0.74	9.4	314
		9.5	7.8	0.42	9.3	325
557	4.86	10.1	9.1	0.29	11.9	505
		10.4	9.3	0.16	10.8	406
559(1)	4.52	10.9	7.5	0	3.8	129
581	4.22	11.6	8.5	0.57	6.7	226
559(2)	4.99	13.0	12.8	3.7	7.8	314
		13.8	12.9	4.4	7.4	300
562	5.15	14.4	10.9	5.2	13.8	384
555	5.11	15.4	13.0	1.6	6.8	379
		15.4	12.5	2.8	7.3	332
576	5.19	15.6	11.5	2.1	2.0	94
672	3.80	23.4	21.8	5.8	2.6	156
673	3.79	25.0	19.3	6.3	3.7	165
674	4.30	32.5	30.5	7.8	3.5	267
Average*	4.72±.71	14.8±7.22	12.5±6.86	2.6±2.69	7.4±4.01	271±107.9

* In the case of the four individuals for which duplicate determinations are recorded, the average of the pairs of determinations was used in computing the over-all averages.

The Effects of Pupal Mass and Metabolic Rate on the Cycle of Carbon Dioxide Release

1. *Effects of pupal mass*

Among the various factors which were studied in relation to the cyclic release of carbon dioxide, the first to be examined in detail was the insect's size or mass. As illustrated in the typical results recorded in Table II, it was noted as a general principle that large pupae give off larger and less frequent bursts than do smaller pupae of similar metabolic rate.

2. *Effects of the absolute level of metabolism*

Figure 3 decribes the relation between metabolic rate and the four parameters of the cycle of carbon dioxide release in a homogeneous series of pupae of approximately the same mass (4 to 6 grams each). To obtain animals with very high metabolic rates, pupae with integumentary injuries were employed (see section 4 below). Each value is an average obtained over at least two cycles.

TABLE II

The cycle of carbon dioxide release of five paired groups of normal diapausing pupae

(*The individuals in each pair have similar rates of oxygen consumption but different weights*)

Animal no.	Weight (grams)	Rate of O_2 consumption (mm^3/gm. live wt./hr.)	Interburst rate of CO_2 output (mm^3/gm. live wt./hr.)	Average cycle length (hours)	Average burst volume (mm^3)	Average burst volume/gm. (mm^3)
562	4.81	8.7	0.46	4.4	179	37
675	6.62	8.9	0	6.5	309	47
718(9)	3.35	12.8	7.5	6.5	52	16
559	4.99	13.0	4.4	7.4	300	60
593	3.47	15.3	5.1	1.7	58	17
555	5.11	15.4	1.6	6.8	379	74
594	2.70	23.4	6.6	1.5	68	25
672	3.80	23.4	5.8	2.6	156	41
591	2.70	28.5	12.6	0.60	23	9
674	4.30	32.5	7.8	3.5	267	63

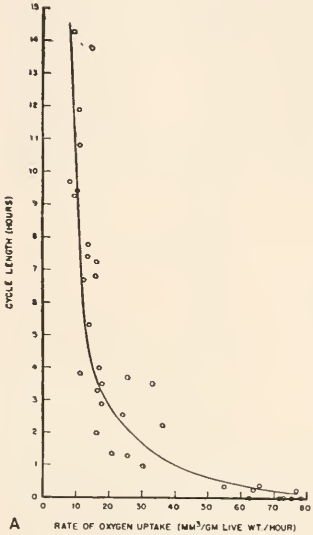
As Figures 3A and B reveal, cycle length and burst volume vary inversely with metabolic rate; *i.e.*, pupae with high metabolic rates are characterized by frequent small bursts. Indeed, it will be noted that when metabolic rate exceeded about 60 mm^3/gm. live wt./hr., many animals gave off their carbon dioxide continuously; however, a few pupae with rates of oxygen uptake as high as 200 mm^3/gm. live wt./hr. showed occasional irregular bursts.

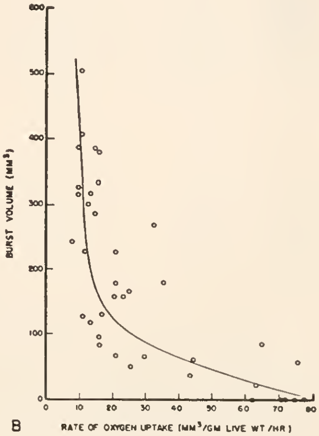
Figure 3C demonstrates the direct proportionality between cycle length and burst volume. This correlation was also seen within the cycles of individual pupae. Figure 3D shows that as the metabolic rate increases, the interburst rate of carbon dioxide output increases.

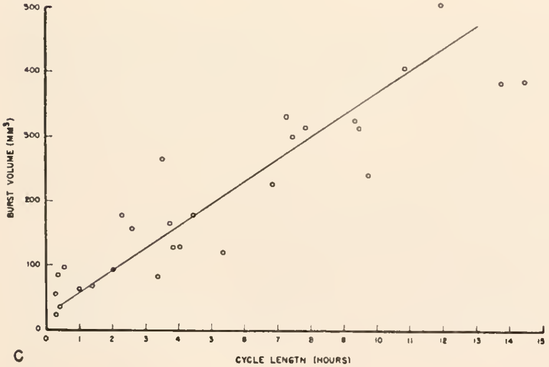
3. *Effects of temperature-induced changes in metabolism*

Results obtained on two groups of diapausing pupae are illustrated in the data summarized in Table III. In five individuals the rates of oxygen consumption and carbon dioxide release were first measured at 25° C.; the insects were then equilibrated for one day at 10° C. and the respiratory measurements repeated at the low temperature. In seven other individuals the measurements were performed only at 10° C. after equilibration at this temperature for two days.

It will be observed that low temperature greatly amplifies the cyclic character of carbon dioxide release. Thus, the individual with the lowest metabolism among those in Table III (No. 572) showed, at 10° C., a calculated burst frequency of







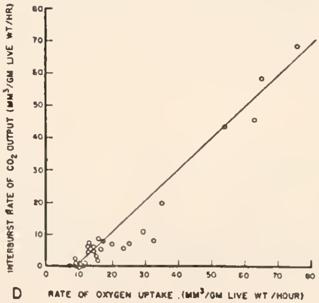


FIGURE 3A. The relation between cycle length and metabolic rate for a series of Cecropia pupae weighing between 4 and 6 grams.

FIGURE 3B. The relation between burst volume and metabolic rate for the same pupae as in 3A.

FIGURE 3C. The relation between burst volume and cycle length for the same pupae as in 3A.

FIGURE 3D. The relation between the interburst rate of carbon dioxide output and metabolic rate for the same pupae as in 3A.

TABLE III

The effect of temperature on the cycle of carbon dioxide release of diapausing pupae

Animal no.	Weight (grams)	Rate of O_2 consumption (mm³/gm. live wt./hr.)		Interburst rate of CO_2 output (mm³/gm. live wt./hr.)		Interburst ratio*		Average cycle length (hours)		Average burst volume (mm³)	
		25°	10°	25°	10°	25°	10°	25°	10°	25°	10°
718(3)	3.87	13.2	2.69	5.7	0.98	50	41	5.3	75** (>58.5)	116	373
718(1)	4.90	20.0	3.42	6.7	0.55	43	23	1.35	25	67	242
718(2)	4.04	15.8	3.62	8.2	1.51	57	42	3.3	20	82	166
718(5)	4.42	16.5	3.62	5.3	0.68	41	21	4.0	23	130	292
718(7)	3.85	16.8	5.30	9.4	1.61	100	40	0	5	0	72
572	4.36	—	1.41	—	0.014	—	1.3	—	160** (>27)	—	764
683	5.25	—	1.57	—	0.061	—	5.0	—	50	—	—
690	5.17	—	1.68	—	0.056	—	4.3	—	65** (>28.5)	—	420
695	5.21	—	1.86	—	0.13	—	8.8	—	50	—	—
706	6.51	—	1.93	—	0.39	—	26.0	—	50	—	—
580	4.80	—	2.01	—	0.58	—	28.0	—	26	—	178
566	5.46	—	3.0	—	0.73	—	27.0	—	17	—	158

* Interburst ratio $= \dfrac{\text{Interburst rate of } CO_2 \text{ output}}{\text{Average overall rate of } CO_2 \text{ output}} \times 100$. This ratio indicates approximately the percentage of carbon dioxide given off continuously.

** In these experiments only one burst was observed and the cycle length was estimated by assuming an over-all R.Q. of 0.78 and a constant interburst rate of CO_2 output. The actual observed minimum values for the cycle length are recorded in parentheses.

one per 160 hours; *i.e.*, about one burst per week. At still lower temperatures to which pupae are normally exposed during winter, one may predict even less frequent release of carbon dioxide—perhaps once in several weeks.

In addition to decreasing the burst frequency, low temperature greatly de-

TABLE IV

The effect of injury on the respiration of six diapausing pupae (Measurements performed at 10° C.)

Animal no.	Weight (grams)	Rate of O_2 consumption (mm³/gm. live wt./hr.)		Interburst rate of CO_2 output (mm³/gm. live wt./hr.)		Average cycle length (hours)		Average burst volume (mm³)	
		Pre-injury	Post-injury	Pre-injury	Post-injury	Pre-injury	Post-injury	Pre-injury	Post-injury
718(3)	3.87	2.7	6.8	0.98	0.49	75	6.7	373	84
718(11)	2.80	3.0	5.7	0.61	0.72	33	7.0	160	95
718(1)	4.90	3.4	8.4	0.55	2.1	25	6.7	242	121
718(5)	4.42	3.6	8.7	0.68	0.79	23	5.8	367	115
718(2)	4.04	3.6	8.6	1.51	3.2	20	2.0	166	28
718(7)	3.85	5.3	11.2	1.61	2.7	5	2.0	89	59

pressed the volume and rate of release of carbon dioxide between bursts; simultaneously the burst volume increased two- to three-fold. One individual (No. 718 (7)) showed continuous carbon dioxide release at 25° C. and cyclic release at 10° C.

4. *Effects of injury-induced increase in metabolism*

Diapausing pupae respond to integumentary injury by a prompt and spectacular increase in oxygen uptake of as much as 8-fold, which then persists for one to several weeks (Schneiderman and Williams, 1953a). By exploiting the injury effect, it was possible to induce major variations in the metabolism of individual animals and to judge the influence on cyclic carbon dioxide release.

Table IV records results obtained in this manner on a series of six animals. The gas exchange was first measured at 10° C. Each pupa was then injured by excising the tip of its abdomen, the resulting defect being sealed with melted paraffin and a plastic slip. After 24 hours at 25° C. the injured pupae, now with enhanced respiration, were returned to 10° C.; 24 hours later, respiratory measurements were performed at the low temperature.

TABLE V

The cycle of carbon dioxide release of an animal during the larval-pupal transformation (An. No. 595; weight = 2.97 gm.)

Days after pupation	−2	1	2	4	6	10	18	34
Rate of O_2 consumption (mm^3/gm. live wt./hr.)	200	131	92	62	47	48	30	15
Interburst rate of CO_2 output (mm^3/gm. live wt./hr.)	158	67	53	11	16	—	9.4	5.8
Average cycle length (hours)	0	.20	.25	.33	.33	.85	1.5	5.5

As is evident in Table IV, the enhanced metabolism was accompanied by a conspicuous decrease in cycle length and burst volume, while in 5 of the 6 animals the interburst rate of carbon dioxide output increased.

In a second series of experiments, the animals after injury were maintained constantly at 25° C. Four days after the injury, the pupal metabolism was maximal and the release of carbon dioxide was continuous. However, after nine further days, the cyclic release of carbon dioxide reappeared as the metabolism decreased towards normal.

5. *Effects of changes in metabolism accompanying the larval-pupal and pupal-adult transformations*

The extremely high metabolism of the mature larva decreases precipitously during the larval-pupal transformation and the early weeks of pupal life (Schneiderman and Williams, 1953a). By the third or fourth week after the pupal moult the metabolism has fallen to the extremely low level characteristic of diapause. These circumstances permitted a further route of approach to the influence of metabolic rate on the cyclic release of carbon dioxide. Observations on a typical animal are recorded in Table V; Figure 4 presents a semi-diagrammatic summary of results obtained from five individuals.

It is evident that carbon dioxide release is continuous throughout the prepupal period. Then, on the first day after the pupal moult, indications of cyclic release appear in the form of frequent bursts superimposed on a large continuous output of carbon dioxide. As the metabolic rate continues to fall, there is a corresponding decrease in the frequency of bursts and in the rate of carbon dioxide release between bursts. These trends continue until the metabolic rate stabilizes at the low diapause level four weeks after pupation.

When previously chilled pupae are placed at 25° C. to terminate the pupal diapause, the increase in metabolic rate after the onset of adult development is accompanied by a reversal of the above-mentioned changes. The carbon dioxide output gradually becomes more continuous and, finally, on about the third day of adult development, the carbon dioxide bursts disappear altogether. Even then, however, the cyclic respiration can be re-established by placing the animals at low temperature.

THE EFFECTS OF VARIATIONS IN OXYGEN AND CARBON DIOXIDE TENSION ON THE CYCLE OF CARBON DIOXIDE RELEASE

The preceding experiments reveal that the mechanism responsible for the discontinuous release of carbon dioxide is profoundly affected by metabolic rate. One would anticipate that an increase in metabolism, by increasing the utilization of oxygen and the production of carbon dioxide, would induce concomitant changes in the tensions of these gases in the respiring cells. This is especially true in an animal such as a silkworm pupa where respiratory movements seem to be absent and where the ventilation of the tracheal trunks apparently depends on gaseous diffusion. For these several reasons it seemed likely that the observed effects of metabolic rate on the cyclic release of carbon dioxide were attributable, in whole or in part, to changes in the "internal" tensions of oxygen and/or carbon dioxide. In order to test this possibility, the internal tensions of these gases were caused to vary.

1. *Effects of oxygen tension on the cyclic release of carbon dioxide*

The gaseous exchange of five diapausing pupae was first measured for 22 hours on successive days at oxygen tensions varying from 6 to 100 per cent of an atmosphere. In this and subsequent experiments employing gas mixtures, each vessel was flushed with about 25 volumes of the experimental gas. This procedure, in itself, commonly caused a slight prolongation of the succeeding cycle of carbon dioxide release; however, the effect was easily recognized and was taken into account in the computations.

The average rate of carbon dioxide output of diapausing Cecropia pupae at 25° C. appeared to be independent of oxygen tension when the latter was varied from 6 per cent to at least as high as 40 per cent of an atmosphere (*cf.* Schneiderman and Williams, 1954). Though failing to affect the over-all metabolism,

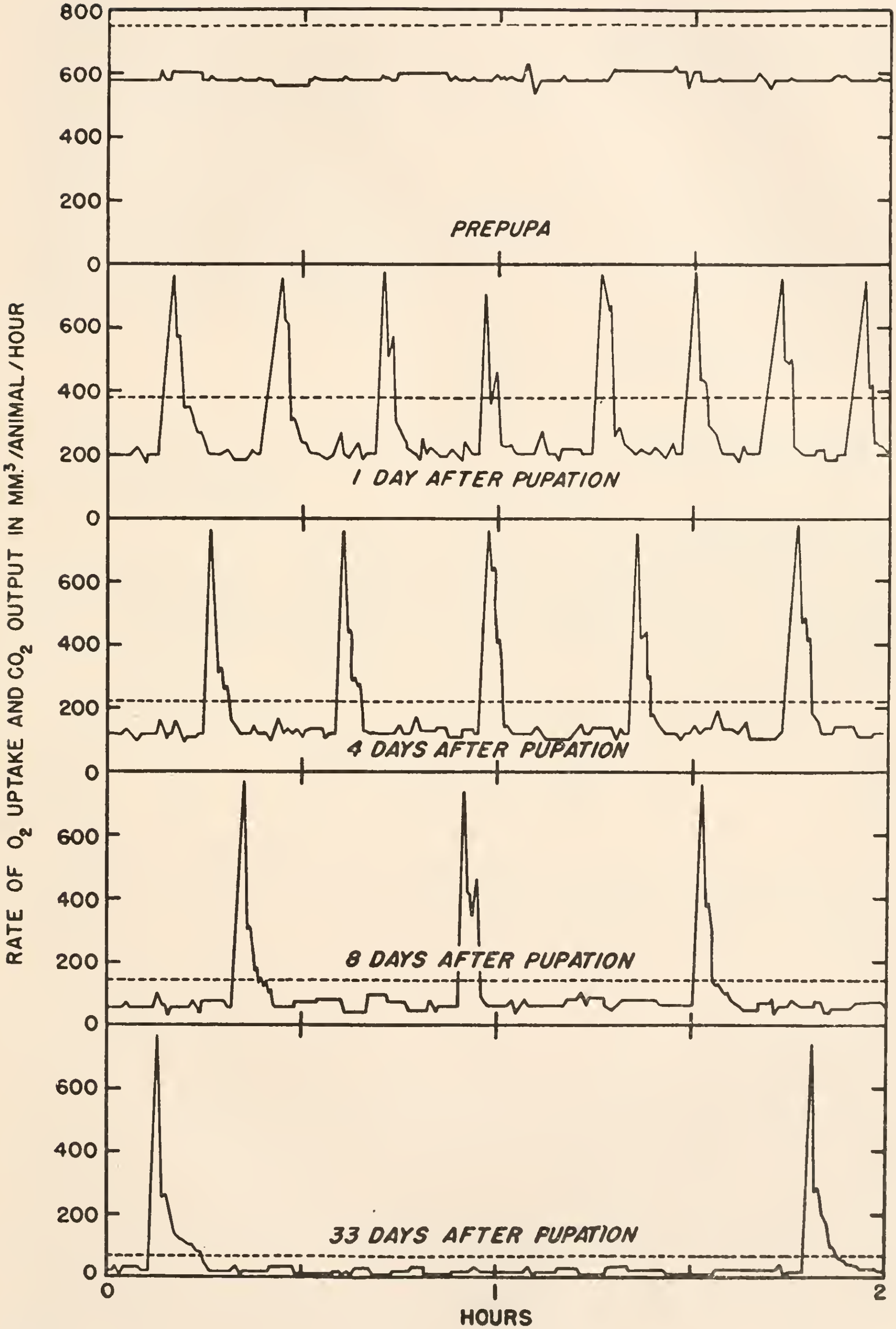


FIGURE 4. The rates of oxygen uptake and carbon dioxide output of a Cecropia silkworm during the larval-pupal transformation (semi-diagrammatic).

oxygen tension nevertheless induced striking changes in the cyclic release of carbon dioxide. Five animals were studied in detail with results summarized in Table VI. It will be observed that, as oxygen tension was decreased below the 21 per cent present in air, the interburst rate of carbon dioxide output increased; *i.e.,* a larger fraction of the carbon dioxide was released continuously. Finally, in 6 per cent oxygen, and occasionally, even in 15 per cent, all of the carbon dioxide was released continuously. When animals were placed in 6 per cent oxygen, it was noted that the rate of carbon dioxide output first increased markedly and then sank to a lower steady level only after several hours. Consequently, it is clear that the low oxygen tension triggered a prolonged period of carbon dioxide release and, in effect, terminated the cyclic release of carbon dioxide with an extremely long and voluminous burst. This same phenomenon has been noted by Buck *et al.* (1953) for pupae placed in nitrogen.

TABLE VI

*The effect of oxygen tension on the cycle of carbon dioxide release of five diapausing pupae**

Animal no.	Weight (grams)	Rate of O_2 consumption (mm^3/gm. live wt./hr.)	Rate of CO_2 output (mm^3/gm. live wt./hr.)					Interburst rate of CO_2 output (mm^3/gm. live wt./hr.)**				
			6%	15%	21% (AIR)	40%	100%	6%	15%	21% (AIR)	40%	100%
718(9)	3.35	12.8	11.0	—	9.8	10.3	—	11.0(100)	—	7.5(39)	4.0(39)	—
718(3)	3.87	13.2	12.1	10.9	11.4	—	—	12.1(100)	8.0(74)	5.7(50)	—	0(0)
718(2)	4.04	15.8	—	14.1	14.3	11.4	—	—	14.1(100)	8.2(57)	3.1(27)	2.6(18)
718(5)	4.42	16.5	14.7	14.9	13.1	12.1	—	14.7(100)	10.9(73)	5.3(91)	1.2(10)	0(0)
718(7)	3.85	16.8	10.4	—	9.4	14.0	—	10.4(100)	—	9.4(100)	3.9(28)	0.8(6)
(continued)			Average cycle length (hours)					Average burst volume (mm^3)				
718(9)			0	—	5.5	7.1	—	0	—	52	150	—
718(3)			0	6.9	5.3	—	7.5	0	76	116	—	—
718(2)			—	2.5	3.3	3.8	7.5	—	0	82	86	—
718(5)			0	8.8	4.0	3.0	5.0	0	158	130	149	170
718(7)			0	0	0	1.9	3.5	0	—	0	76	74

* Each datum is the average of 22 hours of measurements, save in the case of 100% oxygen where measurements were continued for only 7.5 hours.

** The percentage of over-all rate of carbon dioxide output (the interburst ratio) is recorded in parentheses.

It is of particular interest and importance to observe that oxygen tensions above that in air progressively decreased the proportion of carbon dioxide given off continuously. This fact is best appreciated in Table VI in terms of the large and progressive decrease in the percentage of carbon dioxide given off continuously. Indeed, in two of four pupae tested in 100 per cent oxygen, the ratio decreased to zero and there was no detectable release of carbon dioxide between bursts. One individual (No. 718 (7)), which exhibited only continuous release of carbon dioxide in air, showed a typical cycle of carbon dioxide release when placed in 40 per cent oxygen. While decreasing the interburst release of carbon dioxide, high oxygen tensions caused no systematic changes in the frequency or volume of the bursts; this was true save in pure oxygen where the burst frequency was always lower than in air, and where, in some cases, the duration of the bursts increased.

Four of the pupae in this experiment had also been studied in the low temperature study summarized in Table III. A comparison of the results in the two experiments reveals that exposure to 40 per cent oxygen was just as effective as 10° C. in decreasing the rate of carbon dioxide output between bursts. By contrast, the volume and frequency of the bursts were far more sensitive to low temperature than to high oxygen tension.

2. *Effects of carbon dioxide tension on the cyclic release of carbon dioxide*

The respiration of five pupae was measured on successive days over intervals of 15 to 25 hours at carbon dioxide tensions varying from 0 to 15 per cent with results summarized in Table VII.

TABLE VII

*The effect of carbon dioxide tension on the cycle of carbon dioxide release of five diapausing pupae**

Animal no.	Weight (grams)	Rate of O_2 consumption in air (mm^3/gm. live wt./hr.)	Rate of CO_2 output (mm^3/gm. live wt./hr.)					Interburst rate of CO_2 output (mm^3/gm. live wt./hr.)**				
			0 (AIR)	5%	8%	10%	15%	0 (AIR)	5%	8%	10%	15%
675	6.62	8.9	7.1	5.1	5.4	6.0	5.3	0(0)	0.80(16)	2.5(46)	3.9(64)	4.8(90)
558	4.55	9.2	8.2	5.8	7.6	6.4	6.6	2.4(29)	1.8(31)	2.3(30)	—	5.9(90)
673	3.79	25.0	19.2	20.5	21.1	23.2	22.4	6.3(33)	14.7(72)	—	17.0(73)	20.0(90)
670	3.98	29.3	25.9	27.4	23.7	26.1	26.2	10.1(39)	14.7(54)	—	14.3(55)	22.6(86)
674	4.30	32.5	30.4	31.7	28.8	29.4	31.1	12.8(42)	19.7(62)	22.3(77)	23.4(80)	24.3(78)
(continued)			Average cycle length (hours)					Average burst volume (mm^3)				
675			6.5	5.4	.41	.32	.2	309	179	20	20	20
558			14.3	11.9	4.1	.2	.4	386	224	100	20	20
673			3.4	7.2	—	.37	.4	165	246	—	20	20
670			1.6	3.4	—	2.5	.36	116	170	—	116	20
674			3.5	3.3	.4	.31	.31	267	252	40	40	40

* Since it was not feasible to measure oxygen uptakes manometrically in the presence of high concentrations of carbon dioxide, in these calculations we assumed that oxygen uptakes remained constant as carbon dioxide tension varied. Since the average rate of manometer movement for each animal over a long interval did not vary with carbon dioxide tension, such an assumption appeared to be justified.

** The percentage of over-all rate of carbon dioxide output (the interburst ratio) is recorded in parentheses.

At ambient carbon dioxide tensions as high as 5 per cent, the effects on the cycle of carbon dioxide release were trivial: the interburst rate of carbon dioxide output increased slightly, but the burst frequency and volume failed to vary in any systematic way. Increase in carbon dioxide tension to 8 per cent further enhanced the rate of carbon dioxide output between bursts. But the most conspicuous effect of 8 per cent carbon dioxide was a sudden decrease in burst volume and an increase in burst frequency. Both these parameters now showed great irregularities, and in several cases a large and prolonged burst was given off within the first few hours and followed thereafter by a sequence of smaller bursts.

As the carbon dioxide concentration was increased above 8 per cent, more and more of the carbon dioxide was given off continuously. Though periodic irregularities could still be detected, it became difficult or impossible to distinguish between a burst and an interburst. Only one pupa continued to give off large, regular bursts in 10 per cent carbon dioxide. As the carbon dioxide tension was increased to 15 per cent, the carbon dioxide output became almost continuous.

3. *Effects of spiracular occlusion on the cycle of carbon dioxide release*

Pupae of the Cecropia silkworm possess one pair of thoracic spiracles and six pairs of functional abdominal spiracles. These openings are the sole gateways to the tracheal system; consequently, their occlusion affords the simplest possible method of lowering the internal tension of oxygen and elevating the internal tension of carbon dioxide.

The over-all effects of spiracular occlusion were first examined in a series of 14 pupae by measuring the respiratory exchange before and after various numbers of spiracles had been sealed with melted paraffin. Occlusion of the spiracles had similar effects on the over-all uptake of oxygen and output of carbon dioxide. When all spiracles were sealed, the oxygen uptake and carbon dioxide release were reduced to less than 3 per cent; hence, virtually all the gas exchange of the pupa takes place *via* the spiracles. Occlusion of all six pairs of abdominal spiracles depressed the oxygen uptake and carbon dioxide output by one-third. By contrast, occlusion of the thoracic spiracles alone or the three posterior pairs of functional abdominal spiracles had trivial effects on the over-all gas exchange.

The sealing of the three posterior pairs of abdominal spiracles, while failing to lessen the uptake of oxygen, caused noticeable effects on the cycle of carbon dioxide release. In the four individuals studied in detail, there occurred an increase in the interburst rate of carbon dioxide output and a decrease in the burst volume; indeed, in one pupa the bursts disappeared completely. As might be anticipated, it was possible to reverse these effects to a considerable extent by increasing the ambient oxygen tension.

OXYGEN UPTAKE DURING THE CYCLE OF CARBON DIOXIDE RELEASE

In confirmation of the initial observations of Punt (1943, 1948, 1950) the experiments, up to this point, leave little doubt concerning the discontinuous output of carbon dioxide by diapausing Cecropia pupae. However, they likewise direct attention to the paradoxical fact that the oxygen uptake through the very same spiracles is apparently continuous.

Buck *et al.* (1953) suggested that the continuous uptake of oxygen might be an artifact attributable to a change in the volume of the pupa during the interburst period. Such a change in displacement, it was suggested, could result from a utilization of oxygen from the sealed tracheal system leading to a compensatory telescoping of the pupal abdomen. Manometric and volumetric respirometers are, of course, particularly susceptible to errors of this type, and any change in the insect's volume would necessarily be recorded as a net uptake of oxygen. For these several reasons, special attention was centered on the problem of oxygen uptake during the interburst period. Three independent experimental methods were developed as follows:

1. *Divided chamber experiment*

The object of the maneuvers now to be considered was to measure any volume change occurring in the motile collapsible segments of the pupal abdomen while simultaneously measuring the gaseous exchange of the entire pupa. For this purpose the three pairs of functional spiracles on the motile abdominal segments were sealed with melted paraffin. As diagrammed in Figure 5, a plastic diaphragm was then placed around each such pupa just anterior to the motile abdominal segments and sealed in place with melted paraffin. The three individuals treated in this manner continued to show cyclic release of carbon dioxide. Each pupa was then placed in a divided chamber capillary respirometer, adapted from that described by Fraenkel (1932) and diagrammed in Figure 5. The diaphragm separated the two 20-cc. chambers. The posterior chamber enclosing the abdominal segments was filled with water and coupled by a two-hole rubber stopper to a glass capillary calibrated in mm^3 units, the second hole being plugged with a rubber vaccine stopper. The water extended about half-way into the capillary at the outset. In this manner, the posterior chamber and its capillary were made to function as a gauge to measure any changes in the volume of the motile abdominal segments.

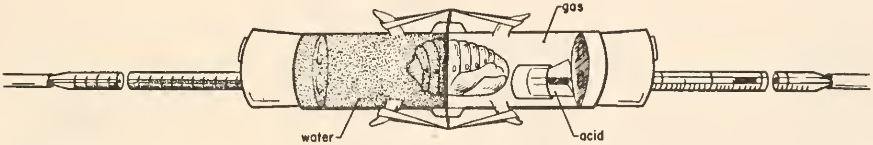


FIGURE 5. Divided chamber capillary respirometer. The diapausing pupa is sealed to a transverse plastic diaphragm so that the insect's anterior end is in the air-filled anterior chamber. The pupal abdomen (with spiracles sealed) is in the water-filled posterior chamber.

The air-filled anterior chamber was similarly coupled by a two-hole rubber stopper to a glass capillary calibrated in 10 mm^3 units. A small paraffin-coated tube was placed in the anterior chamber to separate the insect from a roll of filter paper moistened with 0.3 cc. of HCl. In order to maximize the discontinuous release of carbon dioxide, a mixture of 40 per cent oxygen and 60 per cent nitrogen was flushed through the anterior chamber *via* the vaccine stopper. An index drop of colored detergent solution was then introduced by a long hypodermic needle into the anterior capillary. A short length of rubber tubing was attached to the tips of both measuring capillaries and the entire apparatus finally submerged in a constant temperature bath along with a suitable thermobarometer, the open ends of the rubber tubes being supported above the water level. Simultaneous measurements were made on the two capillaries in order to correlate changes in the displacement of the abdomen with changes in the gas volume attributable to respiration.

A typical experiment is diagrammed in Figure 6. The solid upper curve here records the volume changes of the anterior respiration chamber. The solid lower curve records the volume changes of the abdomen. The dotted line combines these two volume changes and charts the net volume changes that would have been recorded if the animal were enclosed in a Warburg respirometer in the presence of acid. After 105 minutes, oxygen was injected to restore the original gas concentration and the experiment continued.

It is at once evident that the changes which were actually observed in the volume of the abdomen were extremely minor. In the experiment in Figure 6 three of the abdominal volume changes more or less correlate with carbon dioxide bursts. However, bursts occurred on three other occasions without any detectable change in abdominal volume. Moreover, in all cases where changes in abdominal volume were recorded, they took place at least several minutes after the initiation of a burst and were of exceedingly short duration; namely, an abrupt slight decrease followed by an equally rapid increase. It is of particular interest to note that any decrease in abdominal volume was always accompanied by a corresponding increase in the volume of the anterior end. This fact accounts for

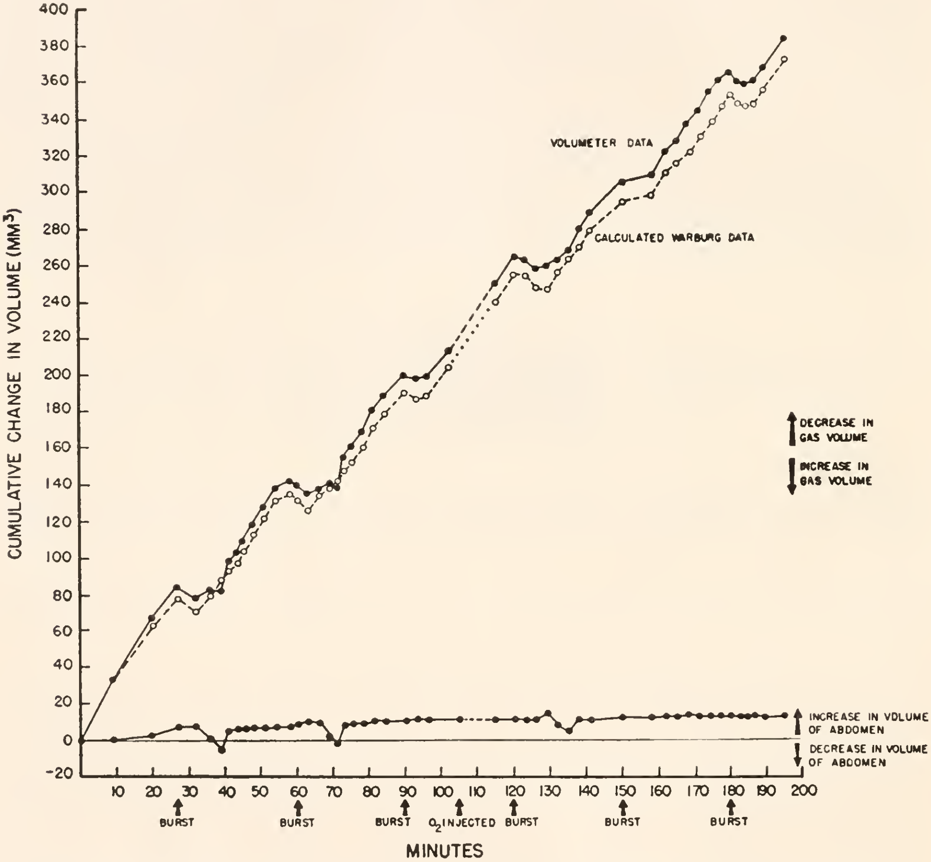


FIGURE 6. Volume changes of the pupal abdomen in relation to the cyclic release of carbon dioxide. See text.

the smooth contours of the dotted curve in Figure 6 as contrasted to the irregularities in the upper solid curve. In short, we were unable to detect any net changes in the displacement of the pupal abdomen during the cycle of carbon dioxide release; evidently, the observed minor alterations in abdominal volume are to be accounted for in terms of the transfer of gas or liquid within the insect by slight changes in the tone of the abdominal muscles.

2. *Projection studies*

By an application of the projection technique of Plateau (1884) and Langendorf (1883), a pupa was placed on a firm pedestal in a dark-room and oriented in the path of the intense point-source of light from a 100-watt zirconium arc. The much enlarged shadow of the pupa was then viewed in profile on a white wall four feet away. Outline sketches of the shadow at frequent intervals revealed no detectable changes in the insect's profile as a function of time.

TABLE VIII

The effect of immobilization on the cycle of carbon dioxide release

A

Immotile anterior pupal fragments

Animal no.	Weight (grams)	Rate of O_2 consumption (mm³/gm. live wt./hr.)	Interburst rate of CO_2 output (mm³/gm. live wt./hr.)	Average cycle length (hours)	Average burst volume (mm³)
579(1)	3.38	30.5	3.6	0.35	85
581(1)	3.16	41	15.5	0.50	43
581(2)	3.20	44	14	0.60	60
579(2)	3.34	48	10.6	0.64	62

B

Pupae immobilized with wax "strait-jackets"

Animal no.	Weight (grams)	Rate of O_2 consumption (mm³/gm. live wt./hr.)	Interburst rate of CO_2 output (mm³/gm. live wt./hr.)	Average cycle length (hours)	Average burst volume (mm³)
718(8)	4.86	20.6	9.6	9.9	178
718(6)	4.61	20.8	16.3	9.0	252

3. *Experiments on immobilized pupae*

By surgical techniques described previously (Williams, 1947) a series of four pupae were transected just anterior to the motile abdominal segments. Each of the immotile anterior fragments was then sealed with melted paraffin to a plastic slip. Several weeks later, after the injury-induced metabolism had subsided, the measurements of respiratory exchange demonstrated the usual cycle of carbon dioxide release along with a continuous uptake of oxygen (Table VIII, part A).

A second procedure involved the immobilization of the abdominal segments of normal pupae by means of wax "strait-jackets." Pupae were anesthetized and strips of melted paraffin applied to their abdominal segments until the segments were completely immobile and incapable of telescoping. Care was taken to avoid any damage to the spiracles. Measurements on two such pupae after immobilization are presented in Table VIII, part B. The discontinuous nature of the carbon dioxide output persisted, as did the apparently continuous oxygen uptake.

Consequently, it is evident that the presence of the motile abdominal segments is not prerequisite for the phenomena under consideration.

DISCUSSION

The results just considered confirm the reality of the carbon dioxide bursts and delineate the factors which affect their magnitude and frequency. All conditions which alter the internal tensions of oxygen or carbon dioxide influence the kinetics of the cyclic release of carbon dioxide. Table IX summarizes the direction of these influences. The data suggest that within the insect the normal stimulus for a carbon dioxide burst is either low internal tension of oxygen, high internal tension of carbon dioxide, or a combination of the two. Moreover, bursts appear to be correlated with low metabolic rate. Therefore, in other groups of insects which, because of their high metabolic rate, release their carbon dioxide continuously at ordinary temperatures, it is likely that bursts can be induced by exposure to low temperature. Also, the greater volume of the burst at low temperatures suggests that the threshold concentration of carbon dioxide necessary to trigger a burst is increased at lower temperatures.

TABLE IX

Summary of the effect of various factors on the cycle of carbon dioxide release

Factor	Metabolic rate	Interburst rate of CO_2 output	Cycle length	Burst volume
Increasing pupal mass	Variable	Decreases	Increases	Increases
Increasing temperature	Increases	Increases	Decreases markedly	Decreases
Integumentary injury	Increases	Increases	Decreases	Decreases
Increasing O_2 tension (6% to 100%)	No effect	Decreases markedly	Variable	Variable
Increasing CO_2 tension (0 to 15%)	No effect	Increases	Variable	Variable
Larval-pupal transformation	Decreases	Decreases	Increases	Variable
Pupal-adult transformation	Increases	Increases	Decreases	Decreases
Occlusion of three abdominal spiracles	No effect	Increases	Variable	Decreases
Occlusion of all spiracles	Maximal decrease	None	None	None

The manometric description of the cycle of carbon dioxide release agrees substantially with the results which Punt (1950) obtained by the diaferometric method. Unfortunately, since Punt's experiments were qualitative and involved only measurements of carbon dioxide output, we cannot make any quantitative comparisons. However, even on a qualitative basis, there are a few minor differences between his results and ours. Whereas we recorded an apparently constant rate of carbon dioxide output between bursts, Punt's data show a period of increased carbon dioxide output prior to each burst. But, since the units along his ordinate are not given, we cannot ascertain the amount of carbon dioxide released in this pre-burst period. Under our experimental conditions, we were unable to detect manometrically a pre-burst increase in carbon dioxide. It is likely, therefore, that Punt's pre-burst increases in carbon dioxide are of small magnitude.

A consideration of the effects of low temperature on the cyclic release of carbon dioxide provides insight into the mechanism underlying the bursts. By comparing the carbon dioxide release curves of animals at 10° and 25° C., we see that, for bursts of equal volume, the rate of evolution of carbon dioxide during the burst is substantially the same at the high and the low temperature. The low temperature coefficient, in itself, suggests that the kinetics of the burst is governed by one or more rate-limiting processes of a physical rather than a chemical character.

By contrast with the cyclic release of carbon dioxide, the uptake of oxygen appears continuous even at low temperatures and under conditions of low metabolic rate. The results of the projection studies and of experiments with the divided chamber and with isolated pupal fragments support this conclusion. Also, as the following calculations show, measurements of tracheal volume add further confirmation.

Vacuum extraction of the gases in the tracheal system and dissolved in the tissue fluids revealed the tracheal volume of Cecropia pupae to be considerably less than 10 per cent of the total volume of the insect. Thus, in the absence of a special oxygen-absorbing blood pigment, a Cecropia pupa weighing 5 grams and having a specific gravity of approximately 1, could store within its tracheal system not in excess of 100 mm^3 of oxygen, and dissolved within its tissue fluids less than an additional 100 mm^3 of oxygen. In short, the total "storage" of oxygen could scarcely exceed 200 mm^3 and is probably much less. However, such a pupa may consume more than 600 mm^3 of oxygen between bursts; that is, more than three times as much oxygen as could be stored under optimum conditions. These facts give assurance that oxygen must enter the insect in substantial quantities between bursts.

The manometric data reveal the magnitude of this oxygen uptake during this interburst period: at 25° C., oxygen enters the spiracles at a rate five to twenty times greater than that at which carbon dioxide makes exit; at 10° C., the difference in rate may reach 100-fold. Any theory which accounts for the discontinuous release of carbon dioxide must also provide for the continuous uptake of oxygen and for the quantitative aspects of the cycle.

It is illuminating to calculate the respiratory quotients one would obtain at 10° and 25° C. if the burst phenomenon were not recognized and the interburst carbon dioxide output employed in calculating respiratory quotients. Thus, at 25° C. the average "interburst R.Q." for the first group of animals in Table III is 0.43 with a range of 0.32 to 0.56. At 10° C. for this same group of animals the average is 0.25 with a range of 0.12 to 0.42, while for the second group it is 0.13 with a range of 0.01 to 0.29. The apparent "systematic change in R.Q. with temperature" is clearly an artifact attributable to the discontinuous release of carbon dioxide. Neglect of this factor doubtless accounts for the peculiarly low R.Q.'s usually reported for diapausing insects.

In the hard-bodied pupal insect we have seen that the spiracles provide the sole gateways to the tracheal system and that the sealing of these orifices brings respiratory exchange substantially to a standstill. Consequently, attention focuses on the role of the spiracular mechanism in relation to the cyclic release of carbon dioxide. This matter will be considered in the paper that follows.

SUMMARY

1. The respiration of diapausing Cecropia pupae is remarkable in that metabolic carbon dioxide is retained within the insect and released during brief periods as "bursts." This finding confirms Punt's prior observations on several species of diapausing insects.

2. At 25° C. in 14 pupae with an average weight of 4.7 grams, bursts of carbon dioxide occurred on the average of once every 7.3 hours and the burst volumes averaged 271 mm^3/animal. The average interburst rate of carbon dioxide output was 2.7 mm^3/gm. live wt./hr., *i.e.*, about one-sixth the average rate of oxygen uptake.

3. The utilization of oxygen, unlike the release of carbon dioxide, shows no discontinuities and "direct" Warburg manometric procedures reveal an apparently continuous uptake of oxygen.

4. Several independent lines of experimentation confirm the manometric observations that oxygen uptake by the pupa is continuous and non-cyclic and direct attention to the surprising fact that, in the interburst period, oxygen may enter the insect at many times the rate at which carbon dioxide makes exit.

5. Virtually all respiratory exchange ceases after the spiracles are sealed with wax; the tracheal system and spiracles are therefore the site of both the discontinuous release of carbon dioxide and the simultaneous continuous uptake of oxygen.

6. The cycle of carbon dioxide release is a function of metabolic rate and therefore of temperature. At low metabolic rates the bursts are accentuated. Thus, at 10° C. carbon dioxide is given off only once in several days and the interburst rate of carbon dioxide output may be but 1/100th the rate of oxygen uptake. If the insect's metabolism is increased by integumentary injury, or development, or increase in environmental temperature, the bursts become more frequent and the continuous release of carbon dioxide more pronounced. The burst phenomenon usually disappears when the oxygen uptake rises beyond 160 mm^3/gm. live wt./hour.

7. The bursts also vanish when the external oxygen tension is decreased below 15 per cent or the external carbon dioxide increased above 10 per cent. The interburst rate of carbon dioxide output is especially sensitive to oxygen tension and in pure oxygen the interburst carbon dioxide output may become undetectable.

8. For bursts of equal volume, the rate of release of carbon dioxide during the burst is substantially the same at 10° and 25° C., signifying that the rate-limiting processes in the rapid release of carbon dioxide are of a physical character with low temperature coefficients.

9. The discontinuous release of carbon dioxide is apparently a widespread phenomenon in diapausing pupae. This fact complicates determinations of respiratory quotients and is evidently responsible for the extremely low and apparently erroneous values reported for diapausing pupae.

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CYCLIC CO_2 RELEASE IN DIAPAUSING AGAPEMA PUPAE

JOHN BUCK AND MARGARET KEISTER

One of the most engaging recent problems in insect respiration is posed by the following observations: (1) Certain insects release CO_2 in brief periods of very rapid output ("bursts") alternating with long periods of very slow output (Punt, 1944, 1950). (2) In such insects O_2 uptake is continuous and constant throughout periods much exceeding the length of the burst cycle (Schneiderman and Williams, 1953, 1955). (3) Though the R.Q. may be 0.1 or less during the interburst period (O_2 taken up 10 or more times as fast as CO_2 is released), the R.Q. computed over an entire cycle is similar to that in many insects with continuous CO_2 release (0.78, Schneiderman and Williams).

The continuity of O_2 uptake shows that the cycle cannot be due to any type of rhythmic change in over-all metabolic rate. The conventional over-all R.Q. indicates that the CO_2 released in the burst should be regarded not as gas suddenly generated at that time but as the normal respiratory CO_2, *produced* at a steady rate throughout the cycle but somehow retarded in *escape* except during the brief burst period. Punt had stated that there were no body movements corresponding to the bursts, and had ascribed the rhythmic release of CO_2 to a corresponding rhythm of spiracular activity. He apparently assumed that entry of O_2 would be reduced by the spiracles whenever escape of CO_2 was retarded, which is not true. Whether the CO_2 retention could be accomplished by cyclic spiracular activity *without* interference with O_2 uptake will appear from the following consideration: Assuming that gas transfer is by diffusion, the quantity passing through the spiracles in unit time is given by Fick's Law: $Q = [D(C^o - C^i)A]/L$, where Q is the rate of gas transfer, D is the diffusivity of the gas in question, C^o and C^i, the concentrations outside and inside the spiracle, A, the area of the aperture of the spiracular valve, and L, the length of the valve lumen in the direction of diffusion. Because O_2 and CO_2 travel the same path, pore area and length can be neglected and relative transfer rates will be determined only by diffusivities and concentration gradients:

$$\frac{Q_{O_2}}{Q_{CO_2}} = \frac{k_{O_2}\,(C^o_{O_2} - C^i_{O_2})}{k_{CO_2}\,(C^i_{CO_2} - C^o_{CO_2})}.$$

Since the diffusivities of O_2 and CO_2 differ only in the ratio of 5:4 it is clear that the spiracles could not, by themselves, bring about the observed 10-fold or more disparity in transfer rates of CO_2 and O_2 during interburst. Accordingly it seems necessary to restudy the CO_2 cycle with special reference to spiracular activity, the triggering of the burst, and the interrelations of the variables of the cycle (burst volume, cycle length, interburst release rate).

It is a pleasure to acknowledge the helpful suggestions of Drs. R. Anderson, L. Chadwick, L. Levenbook, A. G. Richards, H. Specht and J. Verduin, and the kindness of Drs. Schneiderman and Williams in providing us with progress reports of their work, and with specimens of *Platysamia* and *Samia*.

MATERIALS AND METHODS

Diapausing 600–1400 mg. pupae of the saturniid moth *Agapema galbina* were used in most of the work, confirmatory tests being made on 3–5 g. pupae of the saturniid *Rothschildia orizaba.* (CO_2 release was also measured in 9 diapausing pupae of the phalaeniid moth *Admetovis oxymoris* over an average of 20 hours per individual without any discontinuity being observed.) *Agapema,* which pupates (in southern Texas) in December and normally emerges in September and October, was studied from January through May in two successive years, and *Rothschildia* which pupates (in southern California) in September and emerges in May and June, was studied in March and April. Since the two batches of *Agapema* differed somewhat in a number of respects, some of the data are given separately by year. Parasitism by larvae of certain flies, prevalent in saturniid pupae, was shown not to affect the results (Buck and Keister, 1955).

Gas exchange was measured at 5-minute intervals throughout periods of up to 72 hours by Warburg's direct method, O_2 uptake in the presence of 10% KOH, and CO_2 release (by difference) in the presence of 5.5% H_2SO_4, the concentration of acid having the same vapor pressure of water as the alkali. The *Agapema* pupae were rested on the insets of 15-ml. flasks, the liquid being in the flask bottoms, whereas *Rothschildia, Samia* and *Platysamia* were tested in 100-ml. flasks with the volume reduced to about 30 ml. with paraffin, and with the liquid in small watchglasses. Manometers were shaken at about 65 cycles per minute for improved temperature control. Because of the necessity of making continuous records of CO_2 output over many hours and the impossibility of measuring O_2 uptake simultaneously by Warburg manometry, O_2 uptake and CO_2 release in a given individual were usually measured on alternate days. For pupae in full diapause, this practice seems justified by the extremely slow rates of change in weight and metabolic rate, and the rate of CO_2 release during the bursts is so much higher than that of O_2 uptake that no appreciable error is introduced in that part of the cycle. However, a proportionally much greater uncertainty is involved in the computed interburst release rates because the CO_2 is appearing at a rate so much lower than the O_2 uptake rate assumed to apply at that time. Each animal was examined for body movement and heartbeat after each test, to make sure it was alive, since notable bacterial gas exchange can be recorded from dead specimens.

In sealing experiments a resin adhesive (Rebel No. 502, Southern Adhesives Corp.) was used, one-half hour in air being allowed for drying. Unless otherwise stated, respiration measurements were made at 25° C. "Q_{O_2}" and "Q_{CO_2}" indicate rates of gas exchange per unit live weight.

RESULTS

1. *General pattern of CO_2 release*

Data from more than 1900 bursts in 124 pupae in which CO_2 release was measured for an average of 109 hours per individual are summarized in Tables I–III, and in Figure 1. Some idea of the degree of variation between *Agapema* pupae in the same batch, and between batches, can be obtained from Table I, which gives the frequency distribution of mean cycle length in the 124 individuals in runs averaging about 8 hours. *Rothschildia* pupae were similarly variable, ranging

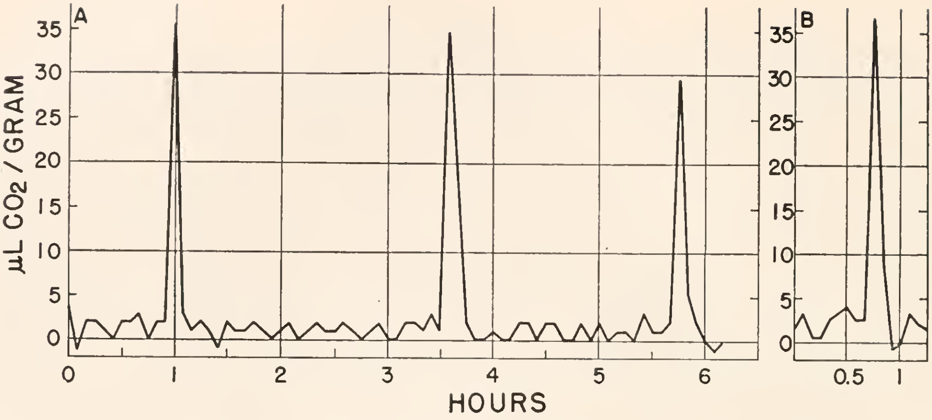


FIGURE 1. Typical records of CO_2 release rate in *Agapema* (A) and *Rothschildia* (B), showing CO_2 bursts; 25°.

from individuals giving small bursts every half hour or so to those giving large bursts at longer than 8-hour intervals. The particular 1953 figures used in Table I cover a period of only a few weeks; later, after various experiments had been run, there had been marked increases in burst frequency in some individuals, several giving bursts as often as every hour, whereas in a few pupae there was a decrease in mean frequency. The 1954 figures involve a longer time span, but in spite of this there was a much larger proportion of long-period pupae than in 1953.

TABLE I

Number of pupae of given mean CO_2 release cycle length at 25°

Batch	Total no. pupae	Mean cumulated hours of obs. per pupa*	Cycle lengths in hours						No bursts
			1–2	2–3	3–4	4–5	5–6	6+	
1953	35	46	0	3	13			17	2
1954	89	47	3	5	3	11	5	60	2

* Ranging from 8–120 hours per individual, but mostly from 20–60 (*e.g.*, the standard deviation for the 1954 pupae was 22.2 hours).

Cycle variation within a given pupa during a period of continuous measurement is indicated by the data for burst volume and cycle length in Table II.

For rough comparison with Schneiderman and Williams' data on the pupa of the Cecropia moth, *Platysamia*, Table III gives the variables of the CO_2 burst cycle in a number of multiple-burst pupae for which corresponding O_2 uptake records were available. From this it appears that *Agapema* pupae differ from those of *Platysamia* in having bursts which are briefer and represent only 16–36 times the volume of CO_2 released continuously in an equal (10 minute) period (rather than up to more than 200-fold) and in releasing only 45–65% of their CO_2 production per cycle as bursts (rather than up to 95%). R.Q.s ranging from 0.4 to 5.0 are obtainable, depending on the period in the cycle chosen for calculation, the average figures for a complete cycle being 0.65 and 0.81 for the small samples treated in Table III. In most of our discussions a value of 0.73 will be assumed.

2. O_2 *uptake in diapausing Agapema pupae*

Concerning the important question of normal O_2 uptake rate, we concluded after careful study that Schneiderman and Williams are correct in reporting a steady and continuous O_2 uptake during periods when CO_2 bursts are occurring. Small but statistically significant perturbations were in fact seen in some of our O_2 uptake records, but can be regarded as artifacts due to inability of the alkali to absorb instantaneously all the CO_2 from bursts occurring just before the time when the manometer was read.

TABLE II

Average CO_2 burst volume and cycle length per individual Agapema pupa at 25°

	No. pupae	No. bursts	Mean burst vol. μL/g.	% volume variation*	No. cycles	Mean cycle length (hours)	% length variation*
1953	9 (22 series)	79	28.6	16.8±2.2	58	3.26	16.0±2.1
1954	29 (42 series)	118	26.1	19.4±2.3	87	4.96	21.2±3.7

* Per cent variation from mean was computed for each series of 2–5 consecutive cycles in one individual. All these were then averaged.

In experiments with various O_2-N_2 mixtures we found Q_{O_2} constant throughout the range 1%–100% O_2. This is important in indicating the unlikelihood of the pupa being hypoxic at any stage in the cycle.

3. *Exclusion of non-spiracular gas-exchange*

In confirmation of Schneiderman and Williams' finding, both O_2 uptake and CO_2 release were practically zero in pupae with all spiracles sealed. This indicates that cutaneous respiration is negligible and that the continuous low-level CO_2 release of interburst must occur via the spiracles, as Punt thought.

Though the anus is not ordinarily functional in lepidopteran pupae we took the precaution of testing the effect of thoroughly sealing the anal end of the pupa.

TABLE III

CO_2 burst cycle variables in Agapema (25°)

(IB = interburst)

	No. pupae	Mean wt.	Q_{O_2} (μL/g./hr.)	Burst duration (min.)	Burst vol. (μL/g.)	Cycle length (hrs.)	IB rate (μL/g./hr.)	Total IB CO_2 per cycle (μL/g.)	Burst rate/ IB rate	Burst as % of Σ_{CO_2} per cycle
1953	13	.85 g.	30	10	33.3	3.6	11.9	42.8	17	44
1954	13	.81 g.	13	14	32.0	4.5	3.8	17.1	36	65

In 15 such pupae 23 bursts occurred in 10 hours, compared with 30 bursts in 14 hours on the preceding day, indicating that intestinal gas is not involved in the CO_2 bursts.

4. *Role of the spiracles*

Since Punt had attributed the cyclic changes in CO_2 release rate to spiracular activity, we investigated this point (*cf.* also Buck, Keister and Specht, 1953). The spiracles of *Agapema* are marked externally by hard, oval stigmata, each of which consists of a rigid thickened border, the peritreme, enclosing a flat immovable plate pierced by a narrow slit with serrated edges. If the stigmal plate is chipped away it can be seen that about 500 μ in from the body surface of the pupa the large tubular atrium is flattened so that the passage is closed. Dissection shows that the flattening is brought about by the apposition of movable sclerotized bars surrounding the atrium in such a way as to make an effective valve (V, Fig. 2). Internal to the valve lies a roughly spherical "manifold" chamber (M) from which a dozen or more large tracheae branch out into the viscera.

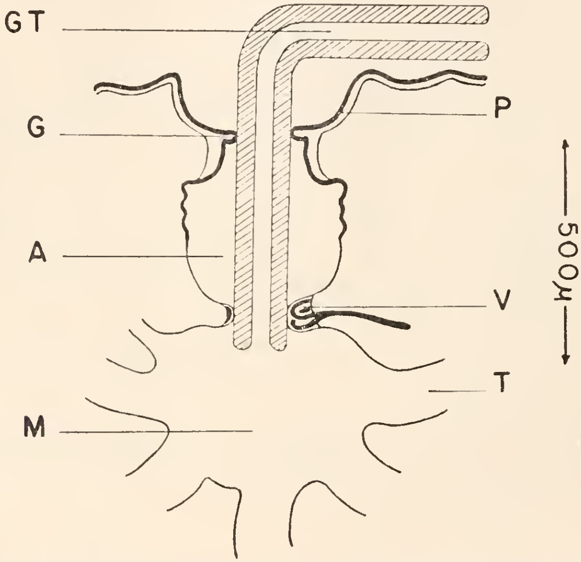
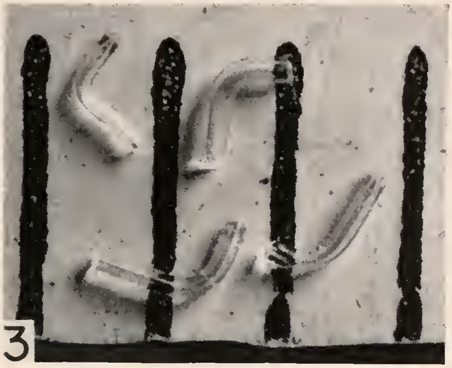
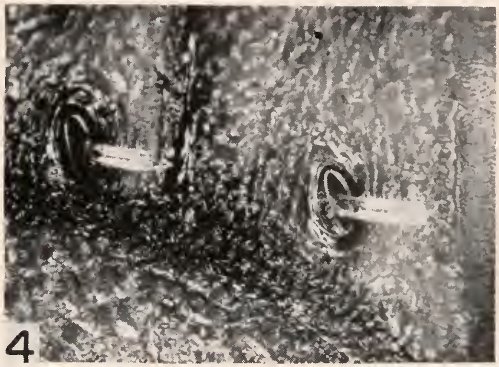


FIGURE 2. Diagram to show glass tube (GT) forced through grating (G) within the peritreme (P) so that it passes through the spiracular atrium (A) and the valve (V) into the tracheal manifold (M).

Under the conditions necessary for observation (removal of peritreme; use of spotlight), the valve opens momentarily to an elliptical slit at intervals considerably shorter than the usual cycle length. It is not known whether this is normal behavior, and it has thus far been impossible to watch the activity of the valves in the living animal during actual respirometry. However, the effect of inactivating the spiracular valve was tested by inserting a capillary glass tube (Fig. 3) through the stigma to a point beyond the valve (Figs. 2, 4, 5). The tube was about 175 μ in outside diameter, fire-polished to prevent injury to the respiratory intima, and provided with a right-angle bend to prevent its penetration deeper than about 600 μ. The lumen of the tubing was about 75 μ, but since the lips of the valve do not close tightly around the cannula a solid rod would probably serve as well. Theoretically a single spiracular tube would be sufficient to keep the tracheal gas in contact with the external environment, since all 14 spiracles are interconnected by large tracheae, but to make sure, 4–6 abdominal spiracles were intubated in each *Agapema* pupa, and 3 in *Rothschildia.*





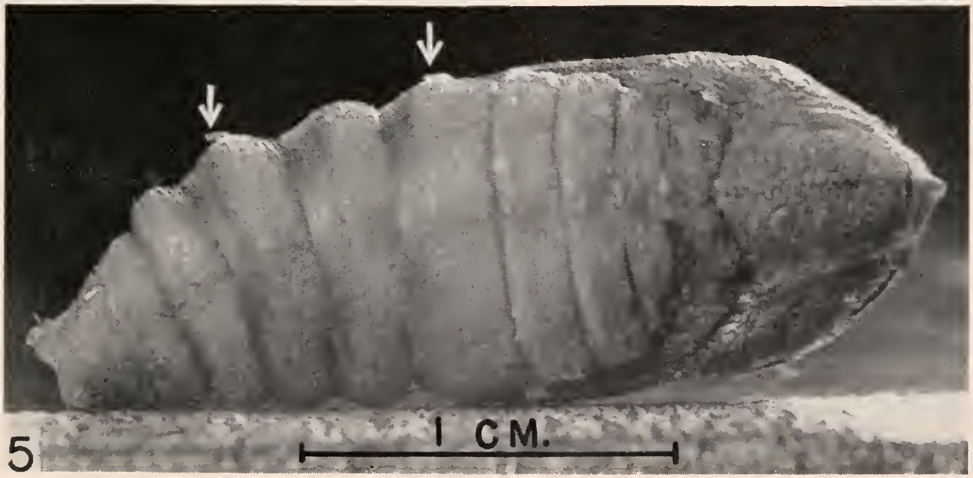


FIGURE 3. Fire-polished glass tubes used for spiracular intubation (on millimeter rule).

FIGURE 4. Tubes in place in 2d and 3d abdominal spiracles of the left side. × 20.

FIGURE 5. Dorsal view of *Agapema* pupa showing tubes in place in 3d and 5th abdominal spiracles of the left side.

The effect of spiracular intubation was very dramatic. In 16 *Agapema* pupae not a single burst was recorded in 7½ consecutive hours, whereas the same individuals had given 38 bursts during 8 hours on the previous day. Confirmatory results were obtained in *Rothschildia.* When the tubes were removed the pupae still produced no bursts, which was disconcerting until it was discovered microscopically that the tubes had warped the valves so that they could no longer close. When, thereupon, only the spiracles which had been intubated were sealed, the capacity to release CO_2 discontinuously was completely restored, and the pupae remained alive and apparently normal for several months thereafter.

5. *Relation of spiracles to water balance*

Since the intubation experiments indicated that the spiracles are concerned with CO_2 retention during interburst, we thought it relevant to see whether the escape of water vapor is likewise impeded. We have not yet been able to measure water loss separately in burst and interburst periods, but over-all rate of loss was readily ascertained from over-all weight loss corrected for loss of respired solids. Figure 6 gives the mean over-all weight loss and mean O_2 uptake rates of each of 3 groups of 5 pupae carefully selected to be practically identical in physical characteristics and burst history, and Table IV gives some average data on weight and water loss. As shown in Figure 6, normal weight loss in laboratory air is almost vanishingly small, amounting to less than 0.5% in 6 days. However, in the 6 days after

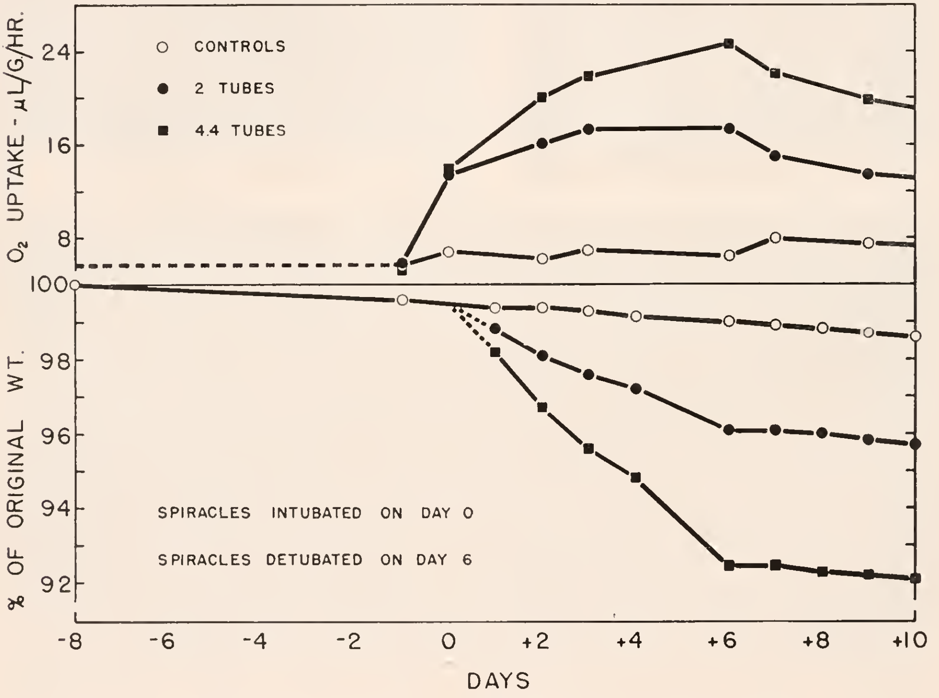


FIGURE 6. Rates of O_2 uptake (above) and weight loss (below) of pupae before, during, and after spiracular intubation, in comparison with controls; 25°.

intubation the group with two spiracles per pupa intubated lost 3.5% of its initial weight, and the group with an average of 4.4 intubated spiracles per pupa lost 7%. When the tubes were removed and those spiracles sealed, the rates of weight loss reverted at once to the control level. The water loss under various conditions, corrected for fat burned (R.Q. taken as 0.73) is given in the last column of Table IV, and indicates that water loss increases roughly in proportion to the number of spiracles intubated.

The O_2 uptake rates given in Figure 6, the measurement of which required a total of 11% of the duration of the weight loss experiments, show that the intubation operation slowly stimulated respiration. This is in accord with Schneiderman and Williams' (1953) findings on the sustained effects of mechanical injury. It might be thought that the weight losses recorded were due to the stimulated respiration, rather than to water loss, but this is contraindicated by the linearity of weight loss in comparison with the decelerating increase in respiration, by the fact that the ratios of increase in O_2 uptake were not the same as the ratios of weight loss in the two experimental groups, and by the immediate return to the control rate of weight loss after detubation and sealing, as contrasted with the slow decline in Q_{O_2}. Furthermore, if possible substrate loss is computed as glucose, and the peak rate of uptake is taken as applying throughout the period of intubation, the loss of solids is only a trifling fraction of total weight loss (Column 9, Table IV). It can be concluded, therefore, that water vapor, as well as CO_2, is normally retained by the spiracles.

TABLE IV

Weight and water loss in intubated pupae at 25°

Group (5 pupae each)	Initial wt./pupa (mg.)	Wt. loss per pupa per day (mg.)	Peak Q_{O_2}/g./hr. (μL)	Total O_2 uptake per day per pupa* (μL)	Total O_2 uptake per day per pupa* (mg.)	H_2O from carbohydrate per pupa** (mg.)	H_2O from fat/pupa** (mg.)	Carbohydrate loss per day per pupa*** (mg.)	Fat loss per day per pupa*** (mg.)	Water loss per per day pupa (from fat) (mg.)
Control	1027	1.0	5.64	139	0.2	0.11	.08	0.2	.07	.93
2 tubes	1078	6.0	17.26	447	.64	.36	.24	0.6	.22	5.8
4.4 tubes	1060	12.7	24.72	629	.9	.51	.34	.84	.31	12.4

* Computed from peak rate.

** *I.e.*, amount of metabolic water produced, assuming pure substrate respired.

*** *I.e.*, computed from over-all O_2 uptake, assuming pure substrate respired.

The very slow rate of rise and fall in Q_{O_2} gives assurance that rise in metabolic rate *per se* cannot be the cause of the sudden abrogation and restoration of ability to produce bursts in the intubation experiment.

6. *Working hypothesis of the CO_2 burst cycle*

Our demonstration that CO_2 retention is abolished when the spiracles are made inoperative does not prove that the spiracles normally control the burst cycle. Since direct observation during respirometry was not feasible we investigated burst volume, cycle length and interburst release rate under different conditions to see whether these variables are interrelated in ways which are compatible with spiracular action.[1] This requires, however, a description of the cycle in relation to possible triggering of its various phases.

A priori the simplest interpretation of the burst cycle is perhaps that the low-level interburst CO_2 release represents leakage through closed or nearly closed spiracles and the burst represents the liberation, at the time of spiracular opening, of the CO_2 which accumulates in excess of that which can leak out. The spiracles would thus act as a sort of safety valve to prevent the internal CO_2 concentration from rising above a certain level. In terms of control, this interpretation of the burst cycle centers in the starting and in the stopping of the burst, events which seem most reasonably attributed to the opening and the closing of the spiracles. Spiracular closing would thus affect burst volume and burst duration, and spiracular opening would determine cycle length.

7. *Interrelations of cycle variables in individuals and populations*

If burst volume, cycle length and interburst release rate change under various conditions, their interrelations should bear not only on spiracular control but on the hypothesis that CO_2 is at least one of the factors controlling spiracular action, and on the rationale of discontinuous CO_2 release *per se*. Thus, for example, if a larger than average burst were released, due to delay in spiracular *closing* or to wider opening than usual during the burst, it might be expected that the succeeding interburst period would be longer than usual because a longer time would be required for the impounding CO_2 to build up the (abnormally low) internal concentration to the level required to trigger spiracular opening. On the other hand, if the burst were larger than average because of delayed spiracular *opening* (*i.e.*, the internal CO_2 concentration rose higher than usual before a burst), it would be expected that larger than usual bursts would be associated with longer than usual *pre*ceding interbursts.

Previous work has not given a clear answer on this point, possibly because of the very small number of records involved. Punt called attention to unusual burst size associated with unusually long *succeeding* interburst period, and Schneiderman and Williams believe that burst volume is correlated with length of *preceding* interburst period. In *Agapema* an analysis was made of over 300 cycles in 18 pupae, occurring in series of 2–9 consecutive cycles per individual, comparing cycles reckoned as a burst plus the interburst immediately preceding, and as the same burst plus the interburst immediately following. There was very considerable variation between and within individuals and no significant difference in mean magnitude was found in interburst length, total interburst volume or total cycle CO_2 release calculated on the two bases. This could mean either that spiracular opening is as variable as spiracular closing or that the two operations are correlated. At any rate, from the practical standpoint a cycle may apparently be defined as a burst plus either the preceding or succeeding interburst. In our computations of total cycle CO_2 output we have arbitrarily used the latter.

Even the most cursory examination shows that there is some regularity in burst volume and cycle length in successive CO_2 release cycles of a given pupa. However, the degree of variation is such, and the difficulty in measuring an adequate number of consecutive cycles in a single pupa is so great, that no statistically valid distinction between the *degree* of variability of the different cycle variables in an individual can be obtained directly. However, an analysis of variance of cycle variables of the 1954 pupae showed that the variance of burst volume is highly significantly less within individual pupae than between mean burst volume in different pupae. In contrast, cycle length, interburst CO_2 release rate and total interburst CO_2 volume all show about as much variation between different cycles of a single pupa as between pupae. This indicates that burst volume is much more constant than cycle length or interburst rate in successive cycles of an individual. Hence the lack of clear association of a burst with either preceding or succeeding interburst period may mean that the internal CO_2 level for spiracular closing varies in parallel with that for opening so as to keep the total amount of CO_2 released in the burst fairly constant, even though the absolute triggering level (and cycle length) varies.

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[1] Burst duration is probably variable also, but the time resolution of the Warburg method is insufficient to detect such differences.

TABLE V

Correlation analysis of burst cycle variables in two samples of Agapema pupae (Q_{CO_2} = *av. rate of* CO_2 *release over whole cycle*)

Pairing	1953 (29 cycles)			1954 (109 cycles)		
	r	t	p	r	t	p
1. Q_{O_2} vs. Q_{CO_2}	.91	11.4	$<.001$	.50	5.97	$<.01$
2. Q_{O_2} vs. IB rate	.60	3.90	$<.001$	.59	7.56	$<.001$
3. Q_{O_2} vs. B vol./g.	.20	1.06	$>.20$	−.20	2.11	$>.02$
4. Q_{O_2} vs. Σ_{CO_2}/cycle	−.20	1.06	$>.20$	−.20	2.11	$>.02$
5. Q_{O_2} vs. cycle length	−.64	4.32	$<.001$	−.54	6.64	$<.01$
6. Q_{CO_2} vs. IB rate	.79	6.7	$<.001$	.59	7.56	$<.01$
7. Q_{CO_2} vs. B vol./g.	.21	1.11	$>.20$	.15	1.57	$>.05$
8. Q_{CO_2} vs. Σ_{CO_2}/cycle	−.02	.104	$>.90$	−.04	.414	$>.60$
9. Q_{CO_2} vs. cycle length	−.62	4.10	$<.001$	−.52	6.3	$<.01$
10. IB rate vs. Σ_{CO_2}/cycle	.40	2.27	$>.02$	.21	2.22	$>.02$
11. IB rate vs. B vol./g.	.02	.104	$>.90$	−.14	1.46	$>.10$
12. IB rate vs. cycle length	−.16	.84	$>.30$	−.21	2.22	$>.02$
13. B vol./g. vs. Σ_{CO_2}/cycle	.30	1.6	$>.10$	.52	6.3	$<.01$
14. B vol./g. vs. cycle length	.16	.84	$>.30$	.28	3.02	$<.01$
15. CO_2/cycle vs. cycle length	.73	4.89	$<.001$	.63	8.4	$<.01$
16. B vol./g. vs ΣIB_{CO_2}	.15	.69	$>.40$	.12	1.25	$>.10$
17. wt. vs. Q_{O_2}	−.15	.70	$>.50$	−.22	1.61	$>.10$
18. wt. vs. absolute burst vol.	.53	2.86	$<.01$	.48	5.66	$<.01$

Turning now to data from populations, Table V summarizes a correlation analysis of burst cycle variables in two batches of *Agapema* pupae. Since only Q_{O_2}, burst volume and cycle length were measured directly, a conservative 1% level of significance seems desirable. With this criterion, there seems to be a significant inverse relation between metabolic rate and cycle length (pairings 5 and 9), the additional CO_2 apparently appearing mainly during interburst (pairings 2, 6) rather than in the bursts (pairings 3, 7). This is consistent with other indications of constancy or independence of burst volume (pairings 11, 13, 16) and with the analysis of variance in individuals. The relation between burst volume and cycle length, an important one (pairing 14), seems to be clear enough in the 1954 population, namely that pupae with larger bursts have longer interbursts. The correlation is even more striking when means from pupae with cycles longer and shorter than 4 hours are compared ($p = < .001$). Nevertheless the lack of correlation in our 1953 population and the non-association between burst volume and Q_{O_2} (pairing 3), each of which is highly correlated with cycle length, are disturbing. Actually, the analysis summarized in Table V may weight the case against association since there are a number of entries for each short cycle pupa and only one for each long cycle one. If means of cycle variables for pupae with cycles shorter than 4 hours are compared with those for pupae with cycles longer than 4 hours, an inverse association between Q_{O_2} and burst volume significant by t test at the 2% level is obtained.

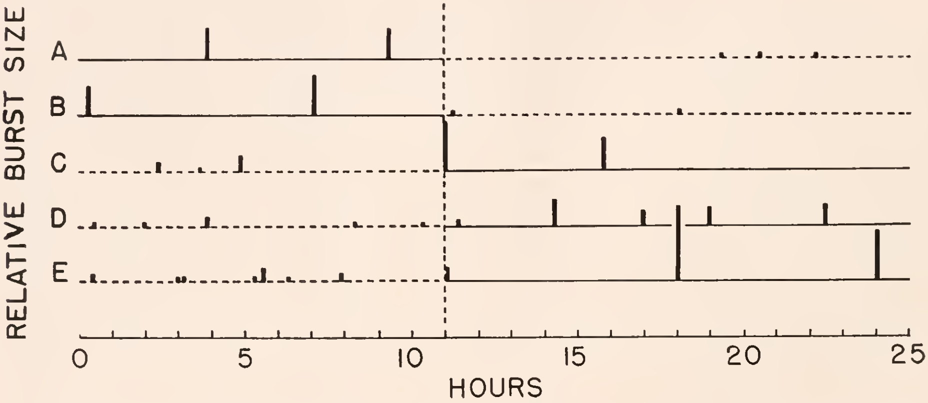

FIGURE 7. CO_2 burst pattern in pure O_2. Shift from air (solid horizontal lines) to pure O_2 (broken horizontal lines) made at 11 hours (broken vertical line). Pupae A and B had been in air with shaker, heaters and lights on for 12 hours (without recording) prior to time zero. Pupae C, D and E had been in O_2 with shaker, heater and lights on for 8 hours prior to time zero. Flasks were not flushed at time zero; 25°.

8. *Effects of temperature and p_{O_2} on the burst cycle*

Punt (1944, 1950) and Schneiderman and Williams (1955) have reported that both increased temperature and increased metabolic rate cause a progressive increase in burst frequency and interburst CO_2 release rate, and a decreased burst volume.

Schneiderman and Williams also reported that increasing the environmental O_2 concentration above that in air caused a progressive decrease in rate of interburst CO_2 release until, in 100% O_2, practically all the CO_2 was released in bursts. Decreasing the ambient O_2 had the opposite effect, bursts disappearing altogether at some concentration between 15% and 6%, *i.e.*, all the CO_2 produced was released continuously. Similar results were obtained with *Agapema,* except with pure O_2, in which bursts were suppressed almost to the vanishing point (Fig. 7). This effect, apparently different from that in *Platysamia,* was found consistently in two 36-hour experiments involving 30 pupae each, and alternated with respect to time of day and sequence of exposure to air and O_2.

No progressive effect of O$_2$ on either cycle length or burst volume is apparent in the records of Schneiderman and Williams nor in *Agapema* population means from different O$_2$ concentrations. The O$_2$ threshold for burst production, which in *Platysamia* lies between 15 and 6%, seems in *Agapema* to be about 10%. Thus, as shown in Table VI, the burst frequency in 13% O$_2$ is indistinguishable from that in air, and bursts are essentially absent in 7%. Of the 30 pupae tested in 10% O$_2$, 13 which had given two or more bursts in a comparable period in air on the preceding day gave no bursts at all, and two others ceased giving bursts in the course of the experiment.

The apparently rather abrupt cessation of burst production at about 10% ambient O$_2$ raises the questions whether there is a real break between continuous and discontinuous types of CO$_2$ release, and if so whether temperature and p_{O_2} differ qualitatively in their effects in this respect. Schneiderman and Williams' 1955 data indicate about 60% higher interburst release rate at 15% O$_2$ than in air, but only three pupae are involved, and the range is above the critical region. In *Agapema* the variability was such that no statistically significant difference in any cycle variable could be established near the "threshold." Some evidence may be provided by the time course of experiments involving prolonged respirometry of multiple-burst pupae in the critical range. Thus Figure 8 shows a decrease in mean burst size of about 2% per hour, cycle length apparently being little affected (no sufficiently long continuous records of O$_2$ uptake were available for computing reliable interburst release rates). The progressive change in burst volume might be attributable either to the computed 2.2% fall in O$_2$ concentration or to the 1.6% rise in CO$_2$ in the flask atmosphere due to pupal respiration during 20 hours. Insofar as this type of evidence is relevant, therefore, there is no indication of a sharp change in mode of CO$_2$ release. However, it should be emphasized that the available temperature and O$_2$ data are too scanty and variable in both *Platysamia* and *Agapema* to give decisive quantitative information.

TABLE VI

Frequency of burst production in different environmental O$_2$ concentrations. Same 30 pupae used in each test; duration of experiments, 18–23 hours; 30°

	13% O$_2$	Air, preceding day	10% O$_2$	Air, preceding day	7% O$_2$	Air, preceding day
No. pupae giving two or more bursts	23	21	5	25	0	27
No. pupae giving single bursts	6	8	10†	3	2‡	3
Total bursts in 18 hours	85	98	30*	135	2	147

† Six occurred within the first hour, hence were probably mechanically triggered (see p. 157).
‡ All occurred within the first hour.
* Almost all were very small.

Further evidence of progressive changes in CO$_2$ retention with decreasing ambient p_{O_2} comes from experiments with pure N$_2$. Since burst production ceases at about 10% O$_2$, no burst would be expected during anoxia. In point of fact all gas exchange did indeed soon cease, but before this happened extra CO$_2$ was evolved

(Buck, Keister and Specht, 1953). Ordinarily this "purge" began when the flasks were flushed with N_2, thus being mainly lost in the equilibration period, but occasionally it was delayed as much as 15 minutes, permitting direct measure (Fig. 9). From such records it was found that purges differ from normal CO_2 bursts in being spread over 31 minutes (mean for 16 purges) and in involving over twice as much CO_2 as a normal burst of the same pupa, even when the flushing is performed immediately after a normal burst in air has occurred. A similar prolonged bleeding off of CO_2 was seen after spiracular intubation. We interpret this to mean that there is normally present in the tissue and blood at all times a large reserve of CO_2 and CO_2 derivatives, and that in a burst the spiracles close again before all this stored CO_2 is released. In an anaerobic environment, or after intubation, on the other hand, the spiracles are forced to stay open, allowing additional CO_2 to diffuse out. Thus the first interburst period in air subsequent to a N_2-induced

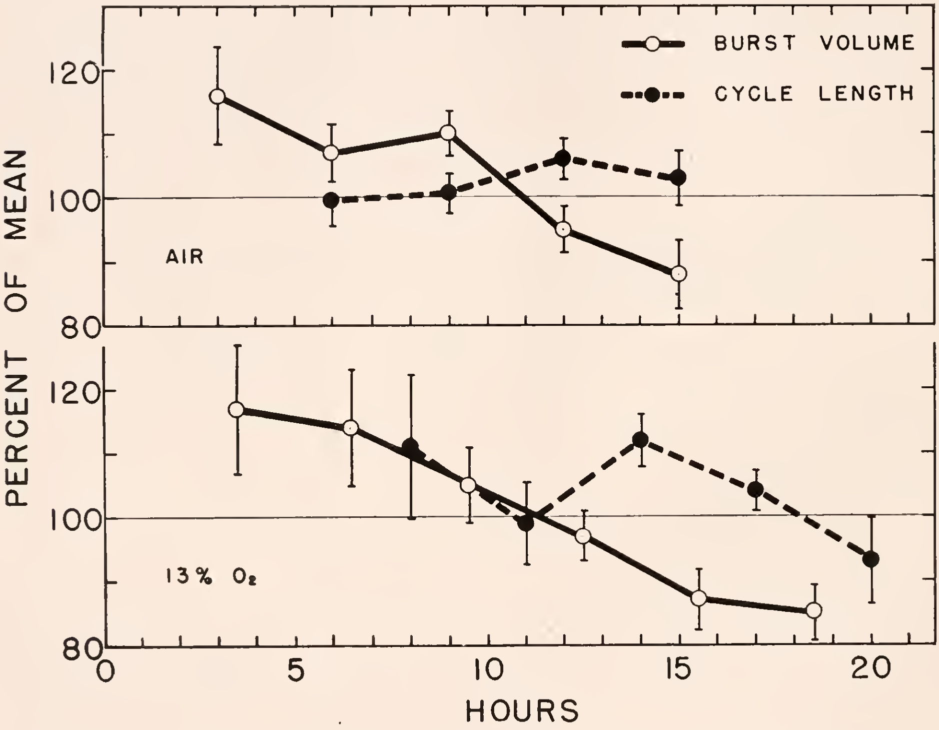

FIGURE 8. Cycle changes in long experiments; 30°. The burst plot was obtained by first plotting, for each pupa, each individual burst volume (as % of mean burst volume for the whole experiment for that pupa) at the time of its occurrence and connecting the points with straight lines. Then the intercepts at the selected 3½, 6½, 9½, etc. hour ordinates with each individual pupal plot were averaged and plotted. The cycle length plot was obtained similarly, plotting the individual cycle lengths (as % of pupal mean) at the time of occurrence of the burst ending the particular interburst period. All pupae gave three or more bursts, and there were 10 pupae in the 13% group and 22 in the control group.

purge, or sealing after detubation, is inordinately lengthened (Table VII), as might be expected if the triggering CO_2 concentration were not attained until both the CO_2 normally involved in the cycle and that in the depleted permanent reserve had been impounded. A similar interpretation might be applied to the casual statement of Punt (1944) that prolonged bursts involve longer than usual cycles.

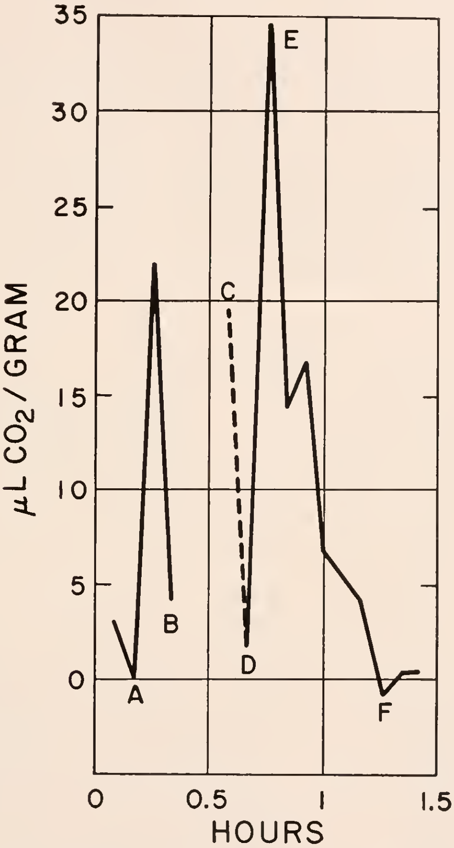

FIGURE 9. CO_2 purge produced by anoxia; 25°. A–B is the normal burst. At B the flask was flushed with pure N_2. C–D is the equilibration from flushing. At D the purge begins, reaching a greater than normal rate at E, and lasting about 3 times as long as the normal burst.

9. *Mechanical triggering of CO_2 release*

Though in normal burst production some internal factor related to respiration triggers the sudden release of CO_2, it was observed that in most experiments a significantly larger number of bursts occurred soon after setup than expected by chance. This is well shown in Figure 10, which gives the time distribution of 90 bursts in 55 records from multiple-burst 1953 pupae in related experiments. Furthermore, 11 of the pupae giving two or more bursts had interburst periods

Table VII

Effects of intubation and N_2 purging on cycle length at 25°

No. pupae	Mean cycle length on preceding day (hr.)	Mean length first cycle after detubation and sealing (hr.)	Mean length first cycle after purge (hr.)	% increase over preceding cycle length	Mean cycle length on day after detubation (hr.)
6*	2.7	4.8		78	2.0
6	4.0		6.2	55	

* Five additional pupae, for which no pre-intubation data were available, showed the sort of post-detubation increase in burst frequency indicated in the last column.

of the proper lengths to reach approximately zero time if extrapolated backwards from the time of occurrence of their ostensible first bursts. Assuming that the first observed bursts of these pupae were in fact their seconds, and that the true first bursts (indicated in solid black in Fig. 10) were lost during the setup and

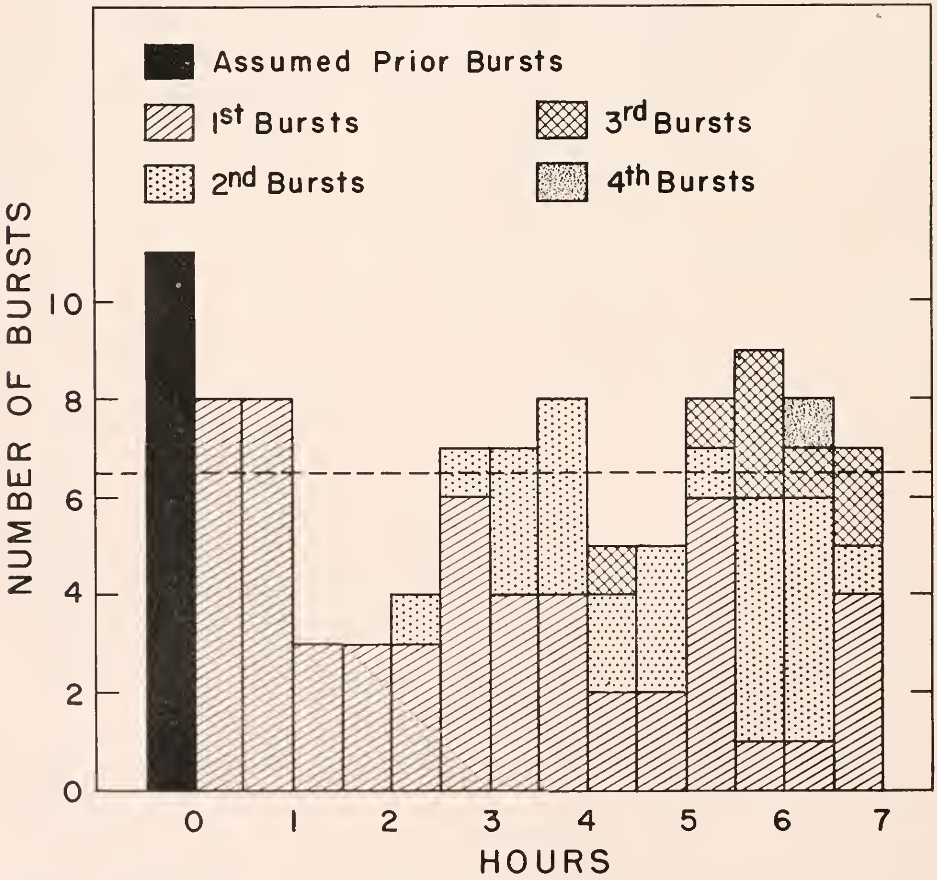

Figure 10. Temporal distribution and sequence of CO_2 bursts to show disproportionate number occurring within the first hour after the start of the experiment; 25°.

equilibration periods at the start of the experiment (the rhythms being "reset" from that point), the discrepancy between the number of bursts occurring during the first hour after setup and during the second becomes even more striking. The 1954 pupae gave a still more exaggerated response, the number of first bursts in the first hour ranging up to 75% in some experiments.

Burst frequency was apparently not correlated with time of day. There was some preponderance of first bursts in the first hour of an experiment in which pupae were left in the flasks all night with lights and shaker on and in which the only apparent environmental change at the start of the experiment was the closing of the manometer cocks. However, a much greater preponderance occurred after initial air-flushing of the flasks and a still greater excess after the usual initial handling of the pupae in weighing, insertion in flasks, etc. It is reasonable that handling might trigger the release of CO_2 because pupae often squirm at this time. However, it is strange that the bursts thus apparently induced can be delayed as much as an hour after the actual handling. At any rate the phenomenon needs to be kept in mind in analyses involving burst frequency and spacing.

DISCUSSION

(a) *The control of CO_2 release and its possible relation to the spiracles*

The intubation experiment shows clearly that spiracular integrity is necessary for the occurrence of the CO_2 burst cycle. The discussion of cycle variations under experimental and metabolic change indicates that many of these responses are at least qualitatively consistent with spiracular involvement in control of CO_2 release. It is therefore reasonable to consider further the triggering of CO_2 release in possible relation to the spiracles.

In our discussion of the cycle as a hypothetical CO_2 regulator it was concluded that the triggering of the burst itself should depend on internal CO_2 concentration. This view is supported strongly by the lengthening of the first interburst period following a N_2 purge or intubation (Table VII), reminiscent of Wigglesworth's (1935) interpretation that the duration of the closed period of flea spiracles is determined in part by accumulation of CO_2. However, the observation that rate of CO_2 release during interburst is constant within the limitations of Warburg respirometry indicates that the change in internal p_{CO_2} during a cycle must be slight. The N_2 "purge" experiment, moreover, shows that much CO_2 remains in the body at the end of a normal burst. How solubility and tissue buffering of CO_2 could permit such relative constancy in the face of burst production will be considered in a separate communication.

The observed changes in CO_2 release rate with changing temperature and ambient p_{O_2} suggest that at least during interburst the aperture of the spiracular valve is influenced also by internal O_2 concentration. Thus circumstances which decrease the availability of O_2, such as a lowering of the environmental p_{O_2}, or increase the utilization of O_2, such as the increased metabolism due to higher temperature or breaking of diapause, would force the spiracles to open wider to provide more O_2. The enlarged opening would allow a more rapid escape of CO_2 which, in turn, would account for the observed increase in rate of interburst CO_2 release with decreased p_{O_2}. The N_2 purge results also fit this scheme, since this maximum ambient O_2 deficiency, presumably inducing an extreme and also sustained opening of the spiracles, could be expected to permit the escape of most of the labile CO_2 in the body.

The possibility that the increased interburst CO_2 release rate with rising temperature is due to the increased rate of CO_2 production, rather than to increased port area, can be neglected if tissue buffers keep intratracheal p_{CO_2} fairly constant, as postulated.

In reference to stimulation of CO_2 release by O_2 deficiency, and also to the question of the progressive nature of the response, it is interesting to recall Schneiderman and Williams' (1955) report that in 6% O_2 ". . . the rate of carbon dioxide release increased markedly and sank to a lower level only after several hours." If the half-hour purge in N_2 (zero O_2) occurs through widely opened spiracles, and if spiracular valve area changes inversely with environmental p_{O_2}, the smaller opening associated with 6% O_2 might indeed be expected to "smear" out the purge over several hours (and, incidentally, to lead to a spurious R.Q. during this period).

Insofar as mode of triggering is concerned, there is ample precedent in other insects for spiracular opening with increased ambient p_{CO_2} (*e.g.*, Hazelhoff, 1926b, 1928), decreased ambient p_{O_2} (Hazelhoff, 1926b; Buck, 1948), or a combination of these gases (Wigglesworth, 1935; Case, 1954). In each instance it may reasonably be assumed that an ambient gas produces its effect via changes in the composition of intratracheal and tissue gas, and is thereby comparable to the triggering due to respiration in diapausing pupae. In view of the well recognized function of spiracles in reducing water loss in insects there is reason to suspect that water vapor also might influence valve activity. Hazelhoff (1926a), however, is reported by Punt (1944) to have found no difference in the opening of the spiracles in adult insects in moist and dry air, and Punt himself found no difference in the pattern of burst production in an adult *Triatoma* under such conditions.

The triggering of CO_2 release in diapausing pupae and its relation to spiracular activity thus emerges as a complex subject. Whatever the mechanism of CO_2 retention it is apparent that the normal cycle involves both an all-or-none type of response, during which occurs the abrupt, extreme and brief increase in CO_2 release rate which we call the burst, and long periods in which the rate is essentially static in spite of increasing retention. As we have seen, the data are insufficient to delineate the transition between the two states. There are, however, two reasons for thinking that the change is not as abrupt as the records suggest. The first is that the transition is partly a matter of the time-resolution of the respirometer. This is well shown by Punt's (1944) records at 22° and 36°, the discontinuity of which would certainly have been lost in Warburg manometry. The second is that if there is a reciprocal relation between ambient p_{O_2} and spiracular aperture it is not a linear one. Thus, from Fick's law, $A \propto \frac{1}{c^o - c^i}$ for constant Q_{O_2} where A = port area and c^o and c^i are the gas concentrations outside and inside. The relation is a rectangular hyperbola and means that at the high end of the p_{O_2} range large changes in O_2 concentration would require only relatively small changes in spiracular aperture, whereas at low O_2 concentrations small changes would induce large changes in spiracular area (causing a spurious break in CO_2 leak rate).

The few interpretable data on triggering could be taken to indicate that CO_2 controls the sudden opening of the spiracles at burst time, while O_2 regulates the valve position sustained during interburst and the rate of over-all release in the range of O_2 concentrations below the burst "threshold." However, in view of Wigglesworth's (1953) and Case's (1954) evidence for interaction of O_2 and CO_2 in stimulating spiracles it seems more likely that the level of tissue CO_2 may not be constant, as assumed in our simplified description of the cycle, but may vary with spiracular area (temperature, p_{O_2}, etc.), hence modifying both interburst release rate and triggering level. The analysis will be carried somewhat further on the theoretical side in another communication.

(b) *Rationale of the CO_2 release cycle*

Insofar as the rate of CO_2 release during interburst and the possible constancy of burst volume in the normal cycle are concerned, the results fit well with the concept of the burst cycle as alternating accumulation and escape of metabolic CO_2. The induced changes in cycle variables, however, are less easily integrated. Cycle length and burst volume do indeed change predictably with temperature, and each shows also an inverse relation with metabolic rate in populations, although this is not necessarily related to the effects of induced changes in individuals. However, the direction of change (decrease with rising temperature) does not seem easily reconcilable with either the anticipated constancy of burst volume or with the expectation that if interburst release rate increases it should take a longer, not a shorter, time to attain the triggering CO_2 concentration. The effects of changes in ambient p_{O_2} are even less well marked and consistent, particularly with concentrations above 21%. We are left, in fact, without a clear picture of either the triggering of the different phases of the cycle, or indeed a satisfactory rationale for the alternating retention and escape of CO_2.

We suggested previously (Buck, Keister and Specht, 1953) that the retention of CO_2 is not the prime objective in itself, but a consequence of provisions for minimizing transpiratory water loss. This idea is consistent with the well recognized role of the spiracles in water conservation in insects in general (*cf.* Hazelhoff, 1926b), with the particularly acute need for conservation in pupae (denied water intake for months or years), and with the demonstrated relation between spiracular integrity and water loss in *Agapema*. Furthermore it could furnish a reason for the sensitivity of the cycle to ambient p_{O_2} in that maximum water retention would require the diffusion port area to be the minimum compatible with adequate respiratory O_2 supply. From Table IV the daily control water loss per one gram pupa is about one mg. or 1360 μL, and the daily CO_2 loss (assuming an R.Q. of 0.73) is $5.64 \times 24 \times 0.73 = 100$ μL, or only $\frac{1}{14}$ of the water vapor loss. Alternatively, if we assume the water vapor loss of the pupae treated in Table III to be in the same proportion to body weight and Q_{O_2} as in those of Table IV, the water loss per cycle would be about 22 and 6 times the observed over-all CO_2 loss in the 1953 and 1954 pupae, respectively. However, the diffusivity of water is only 50% greater than that of CO_2. The validity of viewing the CO_2 release cycle as a consequence of water vapor retention must therefore await the determination of the respective concentration gradients.

Even though a one-mg. daily water loss (0.1% body weight) seems negligible, it would account for an unsupportable loss if maintained through a two-year diapause. Presumably, however, the average rate of desiccation in nature is lower, even in this desert species of moth, because of long periods at temperatures less than 25° and because of maintenance of a higher-than-environmental humidity within the cocoon.

(c) *Mechanism of CO_2 retention*

Evidence has been presented that the spiracles are intimately involved at all stages of the CO_2 release cycle. In controlling the burst *per se* their role presents little difficulty, assuming that tracheal p_{CO_2} is higher than atmospheric, since a sudden enlargement of valve area would force the diffusive loss of any labile CO_2. Our discussion of the Fick equation has shown, however, that the spiracles alone cannot be responsible for the situation in interburst where 10 or more O_2 molecules may enter the pupa for every CO_2 molecule released. The question may thus arise as to whether, in spite of Punt's observations and of other evidence against gross body movements (*e.g.*, Schneiderman and Williams, 1955), gas transfer might be via ventilatory flow rather than by diffusion. This, however, seems excluded by the fact that the CO_2 burst registers manometrically as an actual increase in gas volume in the respirometer flask, whereas an ordinary exhalation, being brought about by decrease in body volume, would merely exchange gas between tracheae and flask without affecting the over-all volume of the system. The possibility of burst production by some sort of biochemical cataclysm, such as sudden acidification of the blood is, in view of the constant presence of a large reservoir of CO_2 (as shown by the purge experiments), highly improbable. From our original discussion of the Fick equation, therefore, we must conclude that rate of CO_2 release during interburst is determined primarily by diffusion gradients. An analysis of this problem, and of the true role of the spiracles in the cycle, will be considered in a later communication.

SUMMARY

1. Forcing a few of the spiracles of the *Agapema* pupa to remain open abolishes the alternate retention and release ("burst") of CO_2 and greatly augments water loss. The effects are reversed by sealing the inactivated spiracles.

2. Pupae exposed to N_2 after a normal CO_2 burst has been produced release an additional volume of CO_2 twice that of the original burst. The first cycle after such a "purge" is nearly twice as long as normal. These results further implicate the spiracles in CO_2 retention and favor the idea that accumulation of CO_2 triggers the burst.

3. A statistical analysis of successive cycles within individual pupae indicates that burst volume tends to be constant, and comparison of individuals in a population shows significant inverse relations between metabolic rate and cycle length, and possibly between burst volume and cycle length. The significance of these findings is discussed in relation to the triggering of CO_2 release and the rationale of the cycle.

4. Mechanical disturbances may also trigger CO_2 bursts.

5. The effects of temperature and ambient p_{O_2} on interburst CO_2 release rate are interpreted in terms of spiracular response to O_2.

6. The transition from cyclic to continuous CO_2 release is discussed in relation to triggering and to spiracular involvement.

7. The prominence of CO_2 in triggering bursts and of the apparent control of the spiracles by O_2 during interburst; the lack of clear functional relation between interburst release rate, burst volume and cycle length; and the lack of special association between burst volume and either preceding or succeeding cycle length, make it difficult to interpret the burst cycle in terms of simple CO_2 regulation, in which the spiracles act as safety valves to prevent undue accumulation of CO_2. It is suggested that the CO_2 release cycle may be a secondary consequence of minimization of transpiratory water loss.

8. Though the spiracles are intimately involved in CO_2 retention and release it is shown theoretically that regulation of valve area *per se* cannot achieve appreciable CO_2 retention without interfering with O_2 uptake. Further analysis in terms of gas diffusion gradients will be discussed elsewhere.

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FIFTY-SEVENTH REPORT, FOR THE YEAR 1954—SIXTY-SEVENTH YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-seventh session of the Laboratory.

The past year appears to have been quite satisfactory as regards the internal operation and scientific services of the Marine Biological Laboratory. The marine biological forms used more extensively by the investigators were abundant. There were few days when collections were impossible due to inclement weather. Two severe hurricanes did not seriously curtail the research and teaching programs as they came late in the season.

1. *Hurricane Damage*

Hurricane Carol hit Woods Hole on August 31 without adequate warning. The resulting high water flooded the basement of the Main Building to about the four-foot level. Through the heroic efforts of the staff and volunteers much valuable equipment was moved to floors above. However some of the heavy apparatus and equipment could not be moved, so suffered seriously in the flood waters. Also the building sustained damage, some of which may become apparent only in the future.

The Laboratory was most fortunate in a grant award of $50,000 by the National Science Foundation which will cover the major share of the uninsurable damage.

2. *Plant Improvements*

Continued progress has been made in the installation of heavier and additional electrical lines in the Main Building to carry the loads required by additional electronic equipment now used by the investigators.

A part of the grant from the National Science Foundation was used for some minor alterations in the buildings which, together with some standby precautions, should prevent a recurrence of damage in the buildings from flood waters of any future hurricanes.

A very pressing problem is the condition of the Crane Building. Built in 1918, this building has undergone serious and unavoidable deterioration in its interior walls, electrical wiring and plumbing. Also the building requires modernization for most effective use in the type of research currently under investigation at the Laboratory. Complete rehabilitation and modernization of this building will cost something of the order of $400,000.

3. *Grants, Contracts and Contributions*

Total income from these sources of support amounted to $97,840.19. This represents 21% of the total Laboratory budget.

Grants:

Rockefeller Foundation, general support	$20,000.00
National Science Foundation	10,000.00
American Philosophical Society	4,300.00
Frank R. Lillie Fellowship	1,500.00
American Cancer Society	6,600.00
National Institute of Health, studies of Arbacia egg and sperm cells	1,080.00
National Institute of Health, encephalization in *Ameiurus nebulosus* during ontogeny	2,012.00
New York Zoological Society	4,000.00

Contracts:

Office of Naval Research, studies in marine biology	15,000.00
Office of Naval Research, studies on marine populations and the effect of environmental factors	4,211.94
Office of Naval Research, function in nerve tissue using the giant axon of the squid	5,941.25
Atomic Energy Commission, studies in radiobiology on the biochemistry of cell nuclei	12,460.00
Atomic Energy Commission, radiobiological studies on the physiology of marine organisms	6,590.00

Contributions:

The annual contribution from the M. B. L. Associates was $3,020, almost double that of the preceding year. This is very encouraging and it is hoped that future contributions will continue to increase. Special contributions were made by Mrs. Edward B. Meigs, Dr. Winterton C. Curtis and Dr. William D. Curtis. Dr. Matilda M. Brooks contributed $2,000 toward the new addition of the M. B. L. Club House in memory of her husband, Dr. Sumner C. Brooks, who was associated for many years with the Laboratory as a member of the staff in the course in Marine Botany, as an investigator, and as a Trustee.

4. *Instruction*

This past year Dr. Harold Bold served very effectively as head of the course in Marine Botany and has consented to continue in that post. Dr. Lewis H. Kleinholz is retiring as head of the course in Invertebrate Zoology having completed a very successful five-year term in this position. He will be succeeded by Dr. Theodore H. Bullock who has spent some summers at the Laboratory as an independent investigator.

5. *Bar Neck Property*

Negotiations on the Bar Neck Property have progressed satisfactorily during the past year and we may reasonably expect that a final settlement of this problem will be achieved in the spring of 1955. The final price which has been tentatively agreed on appears equitable as compared with an appraised value determined for the Laboratory.

6. *Fellowships and Scholarships*

The Laboratory was fortunate in the establishment of three fellowships or scholarships by friends of the Laboratory. These are described in the Annual Announcement. Mrs. Gary N. Calkins established one in memory of her husband, Dr. Gary N. Calkins. Mrs. Kimball Chase Atwood III established one in memory of her mother, Emma Coote Drew and friends and former students of Dr. Edwin Grant Conklin established a scholarship in his memory.

7. *Changes in the By-Laws*

Members of the Corporation should note certain changes in the By-Laws which are included in this Annual Report. These changes appear in Article III, changing the date and time of the Annual Meeting of the Corporation, Article VII, changing the date and time of the Annual Meeting of the Trustees, and Article X, clarifying the relation of the M. B. L. Associates to the Laboratory.

Respectfully submitted,
Philip B. Armstrong,
Director

1. The Staff, 1954

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse

Senior Staff of Investigation

A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati
G. H. Parker, Professor of Zoology, *Emeritus,* Harvard University

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

L. H. Kleinholz, Professor of Biology, Reed College, in charge of course
John H. Lochhead, Associate Professor of Zoology, University of Vermont
Norman A. Meinkoth, Associate Professor of Zoology, Swarthmore College
Grover Stephens, Assistant Professor of Zoology, University of Minnesota
John M. Anderson, Associate Professor of Zoology, Cornell University
Muriel Sandeen, Department of Zoology, Duke University
L. M. Passano, Department of Zoology, University of Washington, Seattle
Morris Rockstein, Department of Physiology, New York University, Bellevue Medical Center

III. Laboratory Assistants

William E. Dossel, Johns Hopkins University
Dorothy M. Skinner, Tufts College

EMBRYOLOGY

I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course
William E. Berg, Assistant Professor of Zoology, University of California, Berkeley
Mac V. Edds, Jr., Associate Professor of Biology, Brown University
John R. Shaver, Assistant Professor of Zoology, University of Missouri
J. P. Trinkaus, Assistant Professor of Zoology, Yale University
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistants

Joan K. Ericksen, Radcliffe College
Roger D. Milkman, Harvard University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California, in charge of course
Stephen Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
George Wald, Professor of Biology, Harvard University
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research
James D. Watson, Senior Research Fellow, California Institute of Technology

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

BOTANY

I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii
Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University, in charge of course
Franz Moewus, Privatdozent, University of Heidelberg and Research Associate, Department of Zoology, Columbia University
Richard C. Starr, Instructor in Botany, University of Indiana

III. Laboratory Assistants

Walter Herndon, Vanderbilt University

IV. Collector

Ann Allen, University of Indiana

MARINE ECOLOGY

I. Consultants

W. C. Allee, University of Florida
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Assistant Professor of Zoology, University of North Carolina

III. Assistant

Edward J. Kuenzler, Department of Biology, University of Georgia

THE LABORATORY STAFF, 1954

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
James McInnis, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Irvine L. Broadbent
Polly L. Crowell
Mrs. Lila Myers
Nancy Shave
Elizabeth Correllus

LIBRARY

Mary E. Castellano, Assistant Librarian
Mary A. Rohan
Naomi Botelhe
Albert Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Arthur Callahan
Robert Gunning
John Head
George A. Kahler
Donald B. Lehy
Alton J. Pierce
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Seaver Harlow
Patricia Philpott

SUPPLY DEPARTMENT

Ruth S. Crowell
Milton B. Gray
Walter E. Kahler
Robert Perry
Patricia M. Conway
Geoffrey Lehy
Robert O. Lehy
Carl Schweidenback
James P. Whitcomb
H. S. Wagstaff

BIOLOGICAL BULLETIN

Donald P. Costello, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
Catherine Henley, Assistant to the Editor

2. Investigators and Students

Independent Investigators, 1954

Allee, W. C., Head, Department of Biology, University of Florida
Allen, M. Jean, Assistant Professor of Zoology, University of New Hampshire
Amberson, William R., Professor of Physiology, University of Maryland Medical School
Ames, Bruce N., Research Fellow, National Institute of Health
Anderson, John M., Associate Professor of Zoology, Cornell University
Armstrong, John C., 1165 5th Avenue, New York City, New York
Armstrong, Philip B., Professor of Anatomy, State University of New York, College of Medicine at Syracuse
Bang, Frederik, Professor of Parasitology, Johns Hopkins University School of Hygiene
Bartlett, James H., Professor of Physics, University of Illinois
Barton, Jay II, Assistant Professor of Zoology, Columbia University
Berger, Charles A., Chairman, Biology Department, Fordham University
Bloch, Konrad, Professor of Biochemistry, Harvard University
Blum, Harold F., Physiologist and Visiting Lecturer, Princeton University
Boettiger, Edward G., Associate Professor of Zoology, University of Connecticut
Bold, Harold C., Professor of Biology, Vanderbilt University
Brown, Frank A., Jr., Professor and Chairman, Department of Biological Sciences, Northwestern University
Butler, Elmer G., Professor of Biology, Princeton University
Cabib, Enrico, Visiting Fellow, Columbia University
Cantoni, G. L., Associate Professor in Pharmacology, Western Reserve University
Carroll, Flora, Independent Investigator, The Institute for Muscle Research
Chang, Joseph J., Research Fellow, Princeton University
Chase, Aurin M., Associate Professor of Biology, Princeton University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Clark, Eliot R., Professor *Emeritus* of Anatomy, The Wistar Institute, University of Pennsylvania
Clement, A. C., Associate Professor of Biology, Emory University
Clowes, G. H. A., Research Director *Emeritus,* Eli Lilly and Company
Cohen, Adolph I., Research Fellow, University of California
Cole, Kenneth S., Technical Director, Naval Medical Research Institute
Collier, Jack R., Graduate Student, University of North Carolina

Cooperstein, Sherwin J., Assistant Professor of Anatomy, Western Reserve University School of Medicine
Cornman, Ivor, Assistant Research Professor in Anatomy, George Washington University
Costello, Donald P., Kenan Professor of Zoology and Chairman, University of North Carolina
Dieter, C. D., Professor of Biology, Director of Pediatric Research, Michael Reese Hospital
Dwyer, John D., Director of Department of Biology, Saint Louis University
Edds, Mac V., Jr., Associate Professor of Biology, Brown University
Elliott, Alfred M., Associate Professor of Zoology, University of Michigan
Ephrussi, Boris, Professor at the Faculté des Sciences, Paris
Eyzaguirre, Carlos, Guggenheim Fellow, Wilmer Institute, Johns Hopkins Hospital
Failla, G., Professor of Radiology, Columbia University
FitzHugh, Richard, Instructor, Wilmer Institute, Johns Hopkins Hospital
Fraenkel, G. S., Professor of Entomology, University of Illinois
Fries, E. F. B., Associate Professor, The City College of New York
Gall, Joseph G., Instructor in Zoology, University of Minnesota
Gasteiger, Edgar L., Associate in Physiology, Harvard Medical School
Gersh, Isidore, Professor of Anatomy, The University of Chicago
Goodchild, C. G. Associate Professor of Biology, Emery University, Georgia
Grant, Philip, Research Associate, The Institute for Cancer Research
Grell, Karl, Professor of Zoology, Max Planck Inst. f. Biologie, Tuebingen, Western Germany
Grosch, Daniel S., Associate Professor of Genetics, North Carolina State College
Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons
Guttman, Rita, Assistant Professor, Brooklyn College
Hamburgh, Max, Research Fellow and Instructor in Anatomy, University of Pennsylvania
Harvey, Ethel Browne, Research Investigator in Biology, Princeton University
Harvey, E. Newton, Professor of Physiology, Princeton University
Hayashi, Teru, Associate Professor of Zoology, Columbia University
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania
Hempling, Harold G., Instructor of Physiology, University of Pennsylvania School of Medicine
Henley, Catherine, Research Associate, University of North Carolina
Hervey, John P., Electronic Engineer, Rockefeller Institute for Medical Research
Hoffman, Joseph F., Lecturer and Research Associate, Princeton University
Holtzer, Howard, Associate in Anatomy, University of Pennsylvania School of Medicine
Hurlbut, William Paul, Assistant, Johns Hopkins University
Hunt, Carlton C., Associate, Rockefeller Institute for Medical Research
Hutter, Otto, Rockefeller Fellow from England, Wilmer Institute, Johns Hopkins Hospital
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania Medical School
Jenkins, George B., *Emeritus* Professor of Anatomy, George Washington University
Jenner, Charles E., Associate Professor of Zoology, University of North Carolina
Johnson, Frank H., Associate Professor of Biology, Princeton University
Johnson, William Harding, Instructor in Physiology, University of Illinois
Karreman, George, Research Associate, Institute for Muscle Research
Kaufman, Seymour, National Institute of Health
Kempton, Rudolf T., Professor and Chairman, Department of Zoology, Vassar College
Keosian, John, Professor of Biology, Rutgers University
King, Thomas J., Research Associate, Institute for Cancer Research
Kleinholz, L. H., Professor of Biology, Reed College
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University
Koch, Henri, Advanced Fellow of the Belgian American Educational Foundation
Krahl, Maurice E., Professor of Physiology, University of Chicago
Kuffler, Stephen W., Instructor, Wilmer Institute, Johns Hopkins Hospital
Kuntz, Eloise, Assistant Professor of Physiology, Vassar College
Lansing, Albert I., Associate Professor in Anatomy, Washington University Medical School
Lauffer, Max A., Head, Department of Biophysics, University of Pittsburgh
Lederberg, Joshua, Associate Professor of Genetics, University of Wisconsin

Levy, Milton, Professor of Chemistry, New York University, Bellevue Medical Center
Lipke, Herbert, Research Associate, University of Illinois
Litt, Mortimer, Research Fellow in Bacteriology, Harvard Medical School
Lochhead, John H., Professor of Zoology, University of Vermont
Loewenstein, Werner, Kellogg Fellow from Chile, Wilmer Institute, Johns Hopkins Hospital
Lorand, Laszlo, Assistant Professor, Wayne University College of Medicine
Lorand, Joyce A., 16834 Evanston, Detroit 24, Michigan
Love, Warner E., Fellow, Johnson Foundation, University of Pennsylvania
Marsland, Douglas, Professor of Biology, Washington Square College
Moewus, Franz, Columbia University
McLaughlin, Jane, Independent Investigator, The Institute for Muscle Research
Meinkoth, Norman A., Associate Professor of Biology, Swarthmore College
Menkin, Valy, Head of Experimental Pathology, Temple University School of Medicine
Metz, Charles B., Associate Professor of Zoology, Florida State University
Middlebrook, Robert, Institute for Muscle Research
Monroe, Clarence L. E., Professor of Biology, Morgan State College
Moore, George M., Professor of Zoology and Chairman of Zoology Department, University of New Hampshire
Moore, John W., Biophysicist, Naval Medical Research Institute
Motomura, Isao, Professor Biological Institute of Tohoku University, Sendai, Japan
Moul, Edwin T., Assistant Professor of Botany, Rutgers University
Nachmansohn, David, Associate Professor of Neurology, College of Physicians & Surgeons
Nanninga, Ludo, Independent Investigator, The Institute for Muscle Research
Nardone, Roland, Assistant Professor of Biology, Catholic University of America
Nelson, Leonard, Assistant Professor of Physiology, University of Nebraska
Osterhout, W. J. V., Member *Emeritus,* Rockefeller Institute for Medical Research
Parpart, Arthur K., Chairman, Department of Biology, Princeton University
Passano, Leonard M., Instructor of Zoology, University of Washington
Person, Philip, Capt., Dental Corps, Walter Reed Army Medical Center
Pettibone, Marian H., Assistant Professor of Zoology, University of New Hampshire
Philpott, Delbert, Independent Investigator, The Institute for Muscle Research
Pick, Joseph, Associate Professor of Anatomy, New York University College of Medicine
Pierce, Madelene E., Professor of Zoology, Vassar College
Proctor, Nathaniel K., Associate Professor, Morgan State College
Ray, Charles, Jr., Assistant Professor of Biology, Emory University
Ray, David T., Instructor of Zoology, Howard University
Rieser, Peter, Research Associate, University of Pennsylvania
Rockstein, Morris, Assistant Professor of Physiology, New York University, Bellevue Medical Center
Ronkin, R. R., Associate Professor of Biological Sciences, University of Delaware
Rose, S. Meryl, Professor of Zoology, University of Illinois
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Sager, Ruth, Assistant, Rockefeller Institute for Medical Research
Sandeen, Muriel I., Instructor in Zoology, Duke University
Scharrer, Ernst, Associate Professor of Anatomy, University of Colorado, School of Medicine
Scharrer, Berta, Assistant Professor, University of Colorado, School of Medicine
Schechter, Victor, Associate Professor of Biology, City College of New York
Schiffman, Gerald, Research Associate, Institute for Muscle Research
Sclufer, Evelyn, National Science Foundation
Scott, Allan, Professor of Biology, Chairman of the Department, Colby College
Scott, Dwight B. McNair, Assistant Professor, Robinson Foundation, University Hospital
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Professor of Zoology, Oberlin College
Shanes, Abraham M., Investigator in Animal Physiology, National Institute of Health
Shaver, John R., Assistant Professor of Zoology, University of Missouri
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa
Smith, A. H. Reesor, Electronics Engineer, Rockefeller Institute for Muscle Research

Sonnenblick, B. P., Associate Professor of Biology, Rutgers University
Speidel, Carl C., Professor of Anatomy and Chairman of Department, University of Virginia, School of Medicine
Spiegel, Melvin, Research Fellow, California Institute of Technology
Starr, Richard, Assistant Professor of Botany, Indiana University
Steele, Richard H., Instructor of Biochemistry, Tulane University
Steinberg, Malcolm S., Graduate Student, University of Minnesota
Stephens, Grover C., Instructor in Zoology, University of Minnesota
Stokey, Alma G., Professor *Emeritus* of Plant Science, Mount Holyoke College
Strehler, Bernard L., Assistant Professor, University of Chicago, Institute of Radiobiology and Biophysics
Stunkard, Horace W., Professor of Biology, New York University
Sturtevant, Alfred H., Professor of Biology, California Institute of Technology
Swift, Hewson H., Assistant Professor in Zoology, University of Chicago
Szent-Gyorgyi, Andrew G., Independent Investigator, Institute for Muscle Research
Szent-Gyorgyi, Albert, Director of Research, Institute for Muscle Research
Szent-Gyorgyi, Eva, Independent Investigator, Institute for Muscle Research
Tracy, Henry C., Associate in Anatomy, University of Mississippi
Trager, William, Associate Member, Rockefeller Institute
Trinkaus, J. P., Assistant Professor of Zoology, Osborn Zoological Laboratory, Yale University
Troll, Walter, Assistant Director and Assistant Professor in Biochemistry, May Institute
Tsuji, Frederick I., Princeton University
Tyler, Albert, Professor of Embryology, California Institute of Technology
deVillafranca, George W., Research Associate, Institute for Muscle Research
Villee, Claude A., Assistant Professor of Biochemistry, Harvard Medical School
Vincent, Walter S., Instructor in Anatomy, State University of New York, School of Medicine
Warner, Robert C., Assistant Professor of Chemistry, New York University, Bellevue Medical Center
Webb, H. Marguerite, Research Associate, Goucher College
Wells, G. P., Reader in Zoology, University College, London
Whiting, P. W., Professor of Zoology *Emeritus*, University of Pennsylvania
Whiting, Anna R., Lecturer in Zoology, University of Pennsylvania
Whittaker, Victor Percy, Assistant Professor of Physiology, University of Cincinnati, College of Medicine
Wichterman, Ralph, Professor of Biology, Temple University
Wilczynski, Jan, Professor of Biology and Genetics, State Lebanese University, Beirut
Wilson, Walter L., Assistant Professor of Physiology and Biophysics, University of Vermont College of Medicine
Wright, Paul A., Assistant Professor of Zoology, University of Michigan
Zwilling, Edgar, Associate Professor, University of Connecticut
Zweifach, Benjamin W., Associate Professor of Biology, New York University, Washington Square College

3. Lalor Fellows, 1954

Ames, Bruce, National Institutes of Health
Collier, Jack R., University of North Carolina
Ephrussi, Boris, Faculté des Sciences, Paris
Ephrussi, Harriet Taylor, Faculté des Sciences, Paris
Gall, Joseph, University of Minnesota
Strehler, B. L., University of Chicago
Vincent, Walter, State University of New York, School of Medicine at Syracuse
Johnson, William Harding, University of Illinois

Beginning Investigators, 1954

Barr, H. Jay, University of Pennsylvania
Bernstein, Gerald S., Lalor Predoctoral Research Fellow, University of Delaware
Borysko, Emil, Damon Runyon Memorial Fund Research Fellow, Johns Hopkins University
Buhite, Robert John, Graduate Fellow, Saint Louis University
Fleischer, Marilyn, Teaching Fellow, New York University
Friedman, Stanley, Research Associate in Entomology, University of Illinois
Fusco, Madeline M., Instructor, Vassar College
Grosof, Jessica, Student, Wellesley College
Hansen, D. D., Grass Trust Fellow, University of California School of Medicine
Haubrich, Robert, Graduate Assistant, University of Florida
Krojanker, Irene, University of Pennsylvania
Landau, Joseph V., Damon Runyon Cancer Research Fellow, New York University
Moos, Carl, Graduate Student, Columbia University
Pepper, Max, Student, University of Pennsylvania School of Medicine
Potter, David D., Graduate Student, Harvard University
Small, Jean E., Graduate Assistant, Brown University
Tunik, Bernard D., Graduate Student, Columbia University
Vennesland, Birgit, University of Chicago
Warren, Martha M., Teaching Assistant in Biology, Brown University
Werntz, Henry Oscar, Graduate Student, Yale University

Research Assistants, 1954

Allen, M. Ann, Indiana University
Barnes, Loy J., University of Missouri
Bennett, Miriam F., Northwestern University
Cahn, Robert David, Swarthmore College
Chalfin, David, Princeton University
Dossel, William E., Johns Hopkins University
Drake, John W., Yale University
Erdman, Howard E., Lehigh University
Erickson, Joan, Radcliffe College
Frost, James L., Johns Hopkins University School of Medicine
Gergen, John A., Harvard Medical School
Gaeuman, John, Oberlin College
Gross, Milton M., State University of New York, College of Medicine at New York City
Groves, Peggy Woford, Yale University
Grundfest, Brooke, Columbia University
Hayward, Hugh R., University of Rochester
Higgins, Don Cheney, State University of New York, College of Medicine at Syracuse
Isenberg, Irvin, University of Chicago
Krupa, Paul L., New York University
LaChance, Leo E., North Carolina State College
Laris, Philip C., Princeton University
Leveque, Theodore F., University of Colorado School of Medicine
Loring, Janet Mason, Harvard Medical School
Mawe, Richard C., Princeton University
Meselson, Matt S., California Institute of Technology
Michaelson, I. Arthur, University of Cincinnati College of Medicine
Milkman, Roger D., Harvard University
Paul, Andrew
Ralph, Charles L., Northwestern University
Shanklin, Douglas R., State University of New York College of Medicine at Syracuse
Shedlovsky, Alexandria, Rockefeller Institute for Medical Research
Shelburne, James Christie, Yale University
Skinner, Dorothy M., Tufts College

Stroud, Agnes N., Argonne National Laboratories
Subtelny, Stephen S., University of Missouri
Sullivan, Robert Little, North Carolina State College
Szent-Gyorgyi, Marta, Institute for Muscle Research
Voorhees, David B., Yale University
Wellington, Frederica Mercer, Harvard Medical School
Zimmerman, Arthur M., New York University

Library Readers, 1954

Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Beloff, Ruth H., Graduate Student, Yale University
Bodansky, Oscar, Professor of Biochemistry, Cornell Medical College
Burwell, E. Langdon, Falmouth Medical Center
Cohen, Seymour S., Associate Professor of Physiological Chemistry and Pediatrics, University of Pennsylvania
Dixon, Frank J., Chairman and Professor of Pathology, University of Pittsburgh
Dorfman, Albert, Associate Professor of Pediatrics, University of Chicago
DuBois, Eugene F., *Emeritus* Professor of Physiology, Cornell University Medical College
Eden, Murray, Special Fellow, Princeton University
Eichel, Herbert J., Research Associate, Hahnemann Medical College
Eisenberg, Max A., Research Associate, Columbia Universtiy
Fitting, Charlotte, Research Fellow in Bacteriology, Harvard Medical School
Freund, Jules, Chief of Division of Applied Immunology, Public Health Research Institute of the City of New York
Friauf, James J., Associate Professor of Biology, Vanderbilt University
Gabriel, Mordecai L., Assistant Professor of Biology, Brooklyn College
Gates, Ruggles, *Emeritus* Professor, University of London, at Harvard University
Ginsberg, Harold S., Associate Professor of Preventive Medicine, Western Reserve University, School of Medicine
Gottschall, Gertrude, Associate, College of Physicians and Surgeons
Greif, Roger L., Assistant Professor of Physiology, Cornell University Medical College
Gudernatsch, Fredrick, Cornell University Medical College
Gurewich, Vladimar, 333 Central Park West, New York 25
Hammond, Warner S., Associate Professor of Anatomy, State University of New York, Upstate Medical Center
Jones, Sarah R., Instructor in Zoology, Connecticut College
Kabat, Elvin A., Professor of Microbiology, College of Physicians and Surgeons
Karush, Fred, Associate Professor of Immunology, University of Pennsylvania School of Medicine
Kimball, Charles P., West Barnstable, Mass.
Kramer, Mollie P., Bibliographer, American Meteorological Society
Levine, Rachmiel, Chairman, Department of Medicine, Michael Reese Hospital
Levy, Arthur L., Research Biochemist, St. Vincent's Hospital
Ling, Gilbert, Assistant Professor of Neurophysiology, University of Illinois, Chicago
Loewi, Otto, Research Professor, New York University College of Medicine
Love, Lois H., National Research Council
Lowenstein, Otto, Research Associate, College of Physicians and Surgeons
McDonald, Sister Elizabeth Seton, Chairman Biology Department, College of Mt. St. Joseph on the Ohio
Meselson, Matt S., Graduate Student, California Institute of Technology
Plough, H. H., Amherst College
Racker, Efraim, Chief, Division of Nutrition and Physiology, Public Health Research Institute
Ratnoff, Oscar D., Assistant Professor of Medicine, Western Reserve University School of Medicine

Root, Walter S., Professor of Physiology, College of Physicians and Surgeons
Rubin, Saul H., Director of Applied Research, Hoffmann-LaRoche Inc.
Shahn, Ezra, Assistant, Sloan Kettering Institute
Shedlovsky, Theodore, Associate Member, Rockefeller Institute
Sonneborn, T. M. Professor of Zoology, Indiana University
Steinhardt, Jacinto, Director Operations Evaluation Group, Navy Department
Sulkin, S. Edward, Professor and Chairman, Department of Microbiology, Southwestern Medical School
Tauber, Hans-Zukas, Assistant Professor of Neurology, New York University College of Medicine
Ulrich, Frank, Research Fellow in Physiology, Yale University School of Medicine
Vishniac, Wolf, Assistant Professor of Microbiology, Yale University
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University
Weber, Annemarie, Research Associate, College of Physicians and Surgeons
Wilson, Irwin B., Assistant Professor of Biochemistry, College of Physicians and Surgeons
Wolff, Walter H., Principal, Wm. C. Bryant High School

Students, 1954

BOTANY

Apollonio, Spencer, Bowdoin College
Corwin, Elizabeth Gayer, Ohio University
Davis, Roberta L., Clark University
Fisher, Mary A., Drew University
Laetsch, Watson M., Wabash College
Larson, Jane L., Clark University
MacFadgen, Josephine Helen, Acadia University
Masiello, Marianna, Hunter College
Pastorius, Janice I., Mount Holyoke College
Redding, William F., DePaul University
Trainor, Francis R., Vanderbilt University
Woodson, Joanna, Woman's College, University North Carolina

EMBRYOLOGY

Baker, K. France, Columbia University
Braunwald, Jack, Amherst College
Bronsweig, Ruth D., University of Florida
Buhite, Robert J., Saint Louis University
Decker, John D., Hartwick College
Defandorf, Virginia, Wesleyan University
Dowling, John A., Harvard College
Fleischman, Julian Burd, Yale University
Fry, Dorothy S., University of Missouri
Giacino, Rosaria Maria, Mount Holyoke College
Grosof, Jessica, Wellesley College
Harris, Joseph David, Purdue University
Izeman, Henry Frank, Brown University
Kallapravit, Boonanake, Washington University
Kavee, Robert C., University of Rochester
Knowles, Robert C., Brown University
Koning, Alice Louise, Iowa State College
McLeod, Guy Collingwood, Waquoit, Massachusetts
Marko, Anita R., Adelphi College
Momberg, Harold Leslie, Missouri University

Nall, Thomas Martin, Wesleyan University
Petersen, Jean Helen, Mount Holyoke College
Roberts, Jane Carolyn, University of Massachusetts
Rondon-Tarchetti, Teresa, Yale University
Shepard, James Edward, Wesleyan University
Szymanski, Christine Dolores, Pennsylvania College for Women
Thurman, Susan Emily, Bryn Mawr
Valerian, Sister M., Marquette University
Widmayer, Dorothea Jane, Wellesley College

PHYSIOLOGY

Ambellan, Elisabeth H., Columbia University
Borenfreund, Ellen, New York University
Brookes, Victor J., University of Illinois
Burgos, Mario H., Harvard Medical School
deFlorida, Francisco A., Tufts College
Donohoo, John T., Notre Dame University
Edwards, Joshua Leroy, Rockefeller Institute for Medical Research
Frost, James L., Johns Hopkins University School of Medicine
Fuller, George Ripley, Cornell Medical College
Hill, Robert Benjamin, Harvard University
Hong, Suk Ki, University of Rochester
Iodice, Arthur Alfonso, Syracuse University
Isaacson, Allen, Harvard University
Kadis, Solomon, Vanderbilt University
Kane, Robert Edward, Johns Hopkins University
Kelly, Arthur Louis, Scripps Institution
Le Roy, Andree, University of Montreal
Levy, Charles Kingsley, University of North Carolina
Littau, Virginia Conway, Columbia University
Mauriello, George E., New York University
Rabinovitch, Michel P., Medical School, University of S. Paulo, Brazil
Santos, Persio de Souza, University of Pittsburgh
Santos, Helen A. L., University of Pittsburgh
Stahl, Franklin William, University of Rochester
Villavicencio, Marino, University of Chicago
Ward, John M., Rutgers University
Wargo, J. Donald, University of Minnesota
Zatzman, Marvin Leon, Ohio State University

INVERTEBRATE ZOOLOGY

Albright, Raymond Gerard, Loyola University
Amenta, Peter Sebastian, Marquette University
Andrus, William DeWitt, Jr., Oberlin College
Arab, Yoosif Mohammed, Johns Hopkins University
Atkin, Adam, Antioch College
Balbinder, Elias, Indiana University
Barki, Gerda S., University of California
Barnett, Audrey Jane, Wilson College
Basch, Paul Frederick, City College of New York
Bauder, Lois Elizabeth, Hiram College
Belden, Don A., Jr., Washington State College
Bender, Marilyn Iris, Hunter College

Blaisdell, Marjorie Ruth, Middlebury College
Botkin, Dorothy Ann, University of Rochester
Brown, Robert Alexander, Harvard College
Burnett, Allison Lee, Cornell University
Chang, Patricia C. H., Johns Hopkins University
Child, Frank Malcolm, III, University of California
Cohen, Matanah, Forest Hills 75, New York
Cooley, Louise M., University of Massachusetts
Davis, Rowland Hallowell, Harvard University
Deck, Lucius Linton, Ford Foundation Grant
Evans, David Robert, George Washington University
Friauf, James J., Vanderbilt University
Genest, Allegra A., Tufts (Jackson College for Women)
Ginsberg, Ellen G., Swarthmore College
Goolsby, Charles M., Harvard University
Gottlieb, Selma G., University of Pennsylvania
Heiman, Emmy Lou, Oberlin College
Hogan, Margaret Rose, Saint Louis University
Hutton, Kenneth Earl, Purdue University
John, Mary C., Fordham University
Joseph, P. V., Fordham University
Kaocharern, Panee, University of Wisconsin
Kaye, Gordon Israel, Columbia University
Kerr, John P., Rutgers University
Kerr, Norman Story, Oberlin College
Lescure, Ono L., Ruxton, Maryland
Littman, David Benjamin, Brooklyn College
Marker, Muriel Josephine, Bard College
McCann, Frances Veronica, University of Illinois
McMillan, Rosamond, Northwestern University
Nawar, Gergis, Washington University
Otero, Luis Raul, George Washington University
Peters, Lewis E., Jr., Purdue University
Quinn, Sister Mary Lorita, University of Notre Dame
Resner, Raymond, Long Island University
Richards, Christina M., University of Illinois
Rohs, Robert Ryan, Massachusetts Institute of Technology
Ryan, Brother Simeon, Saint Louis University
Sage, Janet K., DePauw University
deTerra, Noel, Barnard College
Tomlin, Patricia Ann, Mount Holyoke College
DelVecchio, Robert James, Fordham Graduate School
Vogel, Jhilip H., S.J., West Baden College
Youngpeter, John Michael, Pontiac, Michigan, Senior High School

ECOLOGY

Crowell, Kenneth Leland, Yale University
Eike, Roberta Claire, Elmira College
Gottlieb, Meyer L., 1801 Loring Place, Bronx
Hoffman, Robert Shaw, University of California
Miller, William T., Government of the Virgin Islands
Robinson, Marius, Stanford University
Smith, Ann Haswell, Wesleyan University
Voorhees, David Bruce, Yale University
Wolfe, Margaret K., Harvard University

4. TABULAR VIEW OF ATTENDANCE, 1950–1954

	1950	*1951*	*1952*	*1953*	*1954*
INVESTIGATORS—TOTAL	338	303	306	310	298
Independent	198	186	172	176	180
Under Instruction	43	28	38	37	20
Library Readers	48	37	49	46	52
Research Assistants	49	52	47	51	46
STUDENTS—TOTAL	126	124	123	136	134
Zoology	55	55	55	55	56
Embryology	29	27	23	30	29
Physiology	27	29	27	31	28
Botany	13	13	11	11	12
Ecology			7	9	9
TOTAL ATTENDANCE	464	427	429	446	432
Less persons registered as both students and investigators			2		5
	464	427	427	446	427
INSTITUTIONS REPRESENTED—TOTAL	156	158	149	155	136
By Investigators	114	115	92	90	104
By Students	67	43	57	65	32
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	2	1	1		2
By Students			3	1	1
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	6	8	7	15	11
By Students	2	3	2	6	13

5. COOPERATING AND SUBSCRIBING INSTITUTIONS, 1954

Cooperating Institutions

Amherst College
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph-on-the-Ohio
Columbia University
Columbia University College of Physicians and Surgeons
Cornell University
Cornell University Medical College
Drew University
Duke University
Elmira College
Emory University
Fordham University
George Washington University
Hahnemann Medical School
Harvard University
Harvard Medical School
Institute for Cancer Research
Institute for Muscle Research
Iowa State University
Johns Hopkins University
Johns Hopkins University School of Hygiene
Eli Lilly and Company
Marquette University
Massachusetts General Hospital
Morgan State College
Mount Holyoke College
National Institutes of Health
National Science Foundation
Naval Medical Research Institute
New York University, Heights
New York University College of Medicine
New York University Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oberlin College

Princeton University
Public Health Research Institute of the City of New York
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Sloan Kettering Institute
Southwestern Medical College
State University of New York College of Medicine at Syracuse
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Delaware
University of Florida
University of Illinois
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of North Carolina
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Virginia School of Medicine
University of Wisconsin
Vassar College
Washington University School of Medicine
Wellesley College
Wesleyan University
Western Reserve University School of Medicine
Yale University

Subscribing Institutions

American Meteorological Society
Barnard College
Belgian American Education Foundation
Catholic University of Washington
Egyptian Government Educational Bureau
Embassy of Iraq
Florida State University
Hoffman-La Roche Inc.
Institute of National Education
Long Island University
Massachusetts Institute of Technology
Michael Reese Hospital
Pennsylvania College for Women
Saint Ignatius College
Syracuse University
University of Colorado
University of Mississippi
University of Missouri
University of Nebraska
University of Pittsburgh
Vanderbilt University
Wabash College
Walter Reed Army Medical Center

6. EVENING LECTURES, 1954

June 25
G. B. Baerends .... "The interaction of external and internal stimuli in releasing behaviour."

July 2
S. Meryl Rose .... "Specific inhibition as a factor in cellular differentiation."

July 9
Hewson Swift .... "Cytochemical studies on nucleic acids."

July 16
David R. Goddard .... "Electron transport in plant respiration."

July 23
Britton Chance .... "Dynamics of respiratory enzymes in oxidative phosphorylation."

July 30
Boris Ephrussi .... "The components of the cytochrome-forming system of yeast."

August 6
Franz Moewus .... "Function and biosynthesis of sex hormones in *Chlamydomonas eugametos*."

August 13

H. KOCH	"The mechanism of the active transport of mineral ions by the gill epithelium of the crab *Eriocheir sinensis M.*"

August 20

G. EVELYN HUTCHINSON	"The invasion of fresh water from the seas."

7. TUESDAY EVENING SEMINARS, 1954

July 6

ALLEN GOLDMAN	"Photochemical spectral analysis of the embryonic development of Drosophila."
ROBERTS RUGH and HELEN CLUGSTON	"Radiosensitivity of the various phases of the estrus cycle in the mouse."
MAX LAUFFER, C. C. BRINTON, JR. and ANNE BUZZELL	"The relationship between the surface structure and the electrophoretic mobility of *E. coli* bacteria."

July 13

W. S. VINCENT	"Protein content of starfish nucleoli."
CELIA MARSHAK	"Isolation of nuclei from Asterias eggs in the germinal vesicle stage and their DNA content."
ALFRED MARSHAK	"A theory of the biological role of DNA."

July 20

E. PTEEMANN NIELSON	"Influence of pH on the rate of respiration in Chlorella."
ALBERT TYLER	"Univalent antibodies."
W. TROLL and SOL SHERRY	"The activation of plasminogen by streptokinase."

July 27

JOSEPH PICK	"Sympathectomy in frogs."
O. F. HUTTER and KRISTA KOSTIAL	"The relation of sodium ions to the release of acetylcholine."
BRUNO KISCH	"Electron microscopy of the sarcosomes of the heart."

August 3

P. W. WHITING	"Identifying gene elements in Mormoniella."
RICHARD C. STARR	"Some aspects of sexuality and genetics in desmids."
FRANZ MOEWUS	"On inherited and adapted rutin-resistance in Chlamydomonas."

August 10

D. R. SHANKLIN	"Ionic interaction at Fundulus ectoderm."
DAVID CHALFIN	"Some properties of mature and young erythrocytes."
M. H. JACOBS	"Some new permeability constants for the erythrocyte and their possible significance."

August 17

CARLOS EYZAGUIRRE	"Activation of stretch receptors in Crustacea."
W. R. LOWENSTEIN and O. F. HUTTER	"Sympathetic influence on neuromuscular transmission."
K. S. COLE and J. W. MOORE	"In vivo measurements of the squid giant axon resting and action potentials."

8. MEMBERS OF THE CORPORATION, 1954

1. LIFE MEMBERS OF THE CORPORATION

BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, New York
BILLINGS, MR. R. C., 66 Franklin Street, Boston, Massachusetts
BRODIE, MR. DONALD M., 522 Fifth Avenue, New York 18, New York
CALVERT, DR. PHILIP P., University of Pennsylvania, Philadelphia, Pennsylvania
CARVER, PROF. GAIL L., Mercer University, Macon, Georgia
COWDRY, DR. E. V., Washington University, St. Louis, Missouri
DEDERER, DR. PAULINE H., Connecticut College, New London, Connecticut
DUNGAY, DR. NEIL S., Carleton College, Northfield, Minnesota
GOLDFARB, DR. A. J., College of the City of New York, New York City
JACKSON, MR. CHARLES C., 24 Congress Street, Boston, Massachusetts
JACKSON, MISS M. C., 88 Marlboro Street, Boston, Massachusetts
KING, MR. CHARLES A.
KING, DR. HELEN D., Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania
LEWIS, PROF. W. H., Johns Hopkins University, Baltimore, Maryland
LOWTHER, DR. FLORENCE DEL., Barnard College, New York City, New York
MACNAUGHT, MR. FRANK M., Woods Hole, Massachusetts
MALONE, PROF. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
MEANS, DR. J. H., 15 Chestnut Street, Boston, Massachusetts
MOORE, DR. GEORGE T., Missouri Botanical Gardens, St. Louis, Missouri
MOORE, DR. J. PERCY, University of Pennsylvania, Philadelphia, Pennsylvania
NOYES, MISS EVA J.,
PARKER, DR. GEORGE H., Harvard University, Cambridge 38, Mass.
PAYNE, DR. FERNANDUS, Indiana University, Bloomington, Indiana
PORTER, DR. H. C., University of Pennsylvania, Philadelphia, Pennsylvania
RIGGS, MR. LAWRASON, 120 Broadway, New York City, New York
SCOTT, DR. ERNEST L., Columbia University, New York City, New York
SEARS, DR. HENRY F., 86 Beacon Street, Boston, Massachusetts
SHEDD, MR. E. A.
WAITE, PROF. F. G., 144 Locust Street, Dover, New Hampshire
WALLACE, LOUISE B., 359 Lytton Avenue, Palo Alto, California
WARREN, DR. HERBERT S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania
YOUNG, DR. B. P., Cornell University, Ithaca, New York

2. REGULAR MEMBERS

ABELL, DR. RICHARD G., 7 Cooper Road, New York City, New York
ADAMS, DR. A. ELIZABETH, Mount Holyoke College, South Hadley, Massachusetts
ADDISON, DR. W. H. F., 286 East Sidney Avenue, Mount Vernon, New York
ADOLPH, DR. EDWARD F., University of Rochester, School of Medicine and Dentistry, Rochester, New York
ALBAUM, DR. HARRY G., Biology Department, Brooklyn College, Brooklyn, New York
ALBERT, DR. ALEXANDER, Mayo Clinic, Rochester, Minnesota
ALLEE, DR. W. C., 114 Leigh Hall, University of Florida, Gainesville, Florida

Allen, Dr. M. Jean, Department of Zoology, University of New Hampshire, Durham, New Hampshire

Alscher, Dr. Ruth, Dept. of Physiology, Manhattanville College of the Sacred Heart, Purchase, New York

Amberson, Dr. William R., Dept. of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

Anderson, Dr. J. M., Department of Zoology, Cornell University, Ithaca, New York

Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland

Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania

Armstrong, Dr. Philip B., State University of New York College of Medicine, Syracuse 10, New York

Atwood, Dr. Kimball C., 68½ Outer Drive, Oak Ridge, Tennessee

Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts

Ayers, Dr. John C. Dept. of Oceanography, Cornell University, Ithaca, New York

Baitsell, Dr. George A., 234 Laurence Street, New Haven, Connecticut

Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania

Ball, Dr. Eric G., Dept. of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts

Bang, Dr. F. B., Dept. of Parasitology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland

Barron, Dr. E. S. G., Department of Medicine, University of Chicago, Chicago, Illinois

Barth, Dr. L. G., Dept. of Zoology, Columbia University, New York City, New York

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa

Beck, Dr. L. V., Dept. of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

Beers, Dr. C. D., Dept. of Zoology, University of North Carolina, Chapel Hill, North Carolina

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana

Bernstein, Dr. Maurice, Virus Lab., University of California, Berkeley 4, California

Bertholf, Dr. Floyd M., College of the Pacific, Stockton, California

Bevelander, Dr. Gerrit, New York University School of Medicine, New York City, New York

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts

Bigelow, Prof. Robert P., 72 Blake Road, Brookline 46, Massachusetts

Bishop, Dr. David W., Dept. of Embryology, Carnegie Institute of Washington, Baltimore 5, Maryland

Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland
Bloch, Dr. Robert, Dept. of Botany, University of Pennsylvania, Philadelphia, Pennsylvania
Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey
Bodansky, Dr. Oscar, Dept. of Biochemistry, Sloan-Kettering Division, Cornell University Medical College, New York City, New York
Bodian, Dr. David, Dept. of Epidemiology, Johns Hopkins University, Baltimore 5, Maryland
Boell, Dr. Edgar J., Yale University, New Haven, Connecticut
Boettiger, Dr. Edward G., Dept. of Zoology, University of Connecticut, Storrs, Connecticut
Bold, Dr. H. C., Dept. of Botany, Vanderbilt University, Nashville, Tennessee
Borei, Dr. Hans, Dept. of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania
Bradley, Prof. Harold C., 2639 Durant Avenue, Berkeley 4, California
Bronk, Dr. Detlev W., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York
Brooks, Dr. Matilda M., University of California, Dept. of Physiology, Berkeley 4, California
Brown, Dr. Frank A., Jr., Dept. of Biological Sciences, Northwestern University, Evanston, Illinois
Brown, Dr. Dugald E. S., Dept. of Zoology, University of Michigan, Ann Arbor, Michigan
Brownell, Dr. Katherine A., Ohio State University, Columbus, Ohio
Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland (10405 Muir Place, Kensington, Maryland)
Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia
Bullock, Dr. T. H., University of California, Los Angeles 24, California
Burbanck, Dr. William D., Box 834, Emory University, Georgia
Burdick, Dr. C. Lalor, The Lalor Foundation, Lancaster Pike and Old Baltimore Road, Wilmington, Delaware
Burkenroad, Dr. M. D., 5214 Prytania Street, New Orleans 15, Louisiana
Butler, Dr. E. G., Princeton University, Princeton, New Jersey
Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas
Cantoni, Dr. Giulio, Dept. of Pharmacology, Western Reserve University, Cleveland 9, Ohio
Carlson, Prof. A. J., Dept. of Physiology, The University of Chicago, Chicago 37, Illinois
Carothers, Dr. E. Eleanor, 134 Avenue C, East Kingman, Kansas
Carpenter, Dr. Russell L., Tufts College, Medford 55, Massachusetts
Carson, Miss Rachel, 204 Williamsburg Drive, Silver Spring, Maryland
Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City, New York
Cattell, Mr. Ware, Cosmos Club, Washington 5, D. C.
Chambers, Dr. Edward, Dept. of Anatomy, University of Oregon Medical School, Portland, Oregon

Chambers, Dr. Robert, University Club, Portland, Oregon

Charles, Dr. Donald R., Dept. of Zoology, Division of Biological Sciences, University of Rochester, Rochester 3, New York

Chase, Dr. Aurin M., Department of Biology, Princeton University, Princeton, New Jersey

Cheney, Dr. Ralph H., Biology Department, Brooklyn College, Brooklyn 10, New York

Child, Prof. S. M., Jordan Hall, Stanford University, Stanford, California

Churney, Dr. Leon, Dept. of Physiology, Louisiana State University School of Medicine, New Orleans, Louisiana

Claff, Mr. C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts

Clark, Dr. A. M., Department of Biology, University of Delaware, Newark, Delaware

Clark, Prof. E. R., The Wistar Institute, Woodland Avenue and 36th Street, Philadelphia 4, Pennsylvania

Clark, Dr. Leonard B., Dept. of Biology, Union College, Schenectady, New York

Clarke, Dr. George L., Harvard University, Biological Laboratory, Cambridge 38, Massachusetts

Cleland, Prof. Ralph E., Indiana University, Bloomington, Indiana

Clement, Dr. A. C., Dept. of Biology, Emory University, Emory, Georgia

Clowes, Dr. G. H. A., Eli Lilly and Company, Indianapolis, Indiana

Coe, Prof. W. R., 183 Third Avenue, Chula Vista, California

Cohen, Dr. Seymour S., Dept. of Physiological Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania

Cole, Dr. Elbert C., Department of Biology, Williams College, Williamstown, Massachusetts

Cole, Dr. Kenneth S., Naval Medical Research Institute, Bethesda 14, Maryland

Collett, Dr. Mary E., 34 Weston Road, Wellesley 81, Massachusetts

Colton, Prof. H. S., Box 601, Flagstaff, Arizona

Colwin, Dr. Arthur L., Department of Biology, Queens College, Flushing, New York

Colwin, Dr. Laura H., Department of Biology, Queens College, Flushing, New York

Cooper, Dr. Kenneth W., Department of Biology, University of Rochester, Rochester 3, New York

Cooperstein, Dr. Sherwin J., Dept. of Anatomy, Western Reserve University Medical School, Cleveland, Ohio

Copeland, Dr. D. E., 1027 N. Manchester Street, Arlington 5, Virginia

Copeland, Prof. Manton, Bowdoin College, Brunswick, Maine

Copley, Dr. Alfred L., Chargé de Recherches, Laboratoires de Recherches, Centre International de L'Enfance, Chateau de Longchamp, Bois de Bologne, Paris 16

Cornman, Dr. Ivor, George Washington University, Cancer Clinic, 2300 K Street, N. W., Washington 7, D. C.

Costello, Dr. Donald P., Dept. of Zoology, University of North Carolina, Chapel Hill, North Carolina

Costello, Dr. Helen Miller, Dept. of Zoology, University of North Carolina, Chapel Hill, North Carolina

Crampton, Prof. H. E., American Museum of Natural History, New York City, New York

Crane, Mr. John O., Woods Hole, Massachusetts

Crane, Mrs. W. Murray, Woods Hole, Massachusetts

Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire

Crouse, Dr. Helen V., Goucher College, Baltimore, Maryland

Crowell, Dr. P. S., Jr., Dept. of Zoology, University of Indiana, Bloomington, Indiana

Curtis, Dr. Maynie R., University of Miami, Box 1015, South Miami, Florida

Curtis, Prof. W. C., University of Missouri, Columbia, Missouri

Dan, Dr. Katsuma, Misaki Marine Biological Station, Misaki, Japan

Danielli, Dr. James F., Department of Zoology, King's College, London, England

Dawson, Dr. A. B., Harvard University, Cambridge, Massachusetts

Dawson, Dr. J. A., The College of the City of New York, New York City, New York

Diller, Dr. Irene C., Institute for Cancer Research, Philadelphia, Pennsylvania

Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania

Dodds, Prof. G. S., School of Medicine, West Virginia University, Morgantown, West Virginia

Dolley, Prof. William L., University of Buffalo 14, New York

Donaldson, Dr. John C., University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Doty, Dr. Maxwell S., Department of Biology, University of Hawaii, Honolulu, T. H.

Drinker, Dr. Cecil K., Box 502, Falmouth, Massachusetts

DuBois, Dr. Eugene F., Cornell University Medical College, 1300 York Avenue, New York City, New York

Duggar, Dr. Benjamin M., Lederle Laboratories Inc., Pearl River, New York

Duryee, Dr. William R., Carnegie Institute, 5241 Broad Branch Road, Washington 15, D. C.

Edds, Dr. Mac V., Jr., Dept. of Biology, Brown University, Providence, Rhode Island

Eichel, Dr. Bertram, Bureau of Biological Research, Box 515, Rutgers University, New Brunswick, N. J.

Eichel, Dr. Herbert J., Hahnemann Medical College, Philadelphia, Pennsylvania

Elliott, Dr. Alfred M., Dept. of Zoology, University of Michigan, Ann Arbor, Michigan

Evans, Dr. Titus C., State University of Iowa, Iowa City, Iowa

Failla, Dr. G., College of Physicians and Surgeons, New York City, New York

Fauré-Fremiet, Prof. Emmanuel, Collège de France, Paris, France

Ferguson, Dr. F. P., Dept. of Physiology, University of Maryland Medical School, Baltimore 1, Maryland

Ferguson, Dr. James K. W., Dept. of Pharmacology, University of Toronto, Ontario, Canada

Figge, Dr. F. H. J., University of Maryland Medical School, Lombard and Green Streets, Baltimore 1, Maryland

Fingerman, Dr. Milton, Dept. of Zoology, Newcomb College, Tulane University, New Orleans 18, Louisiana

Fischer, Dr. Ernst, Dept. of Physiology, Medical College of Virginia, Richmond 19, Virginia

Fisher, Dr. Jeanne M., Dept. of Biochemistry, University of Toronto, Toronto, Canada

Fisher, Dr. Kenneth C., Dept. of Biology, University of Toronto, Toronto, Canada

Forbes, Dr. Alexander, Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Fraenkel, Dr. Gottfried S., Dept. of Entomology, University of Illinois, Urbana, Illinois

Fries, Dr. Erik F. B., Dept. of Biology, City College of New York, New York City, New York

Frisch, Dr. John A., Canisius College, Buffalo, New York

Furth, Dr. Jacob, 18 Springdale Road, Wellesley Farms, Massachusetts

Gabriel, Dr. Mordecai, Dept. of Biology, Brooklyn College, Brooklyn, New York

Gaffron, Dr. Hans, Dept. of Biochemistry, University of Chicago, Chicago 37, Illinois

Gall, Dr. Joseph G., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Galtsoff, Dr. Paul S., Woods Hole, Massachusetts

Gasser, Dr. Herbert S., Director, Rockefeller Institute, New York 21, New York

Gates, Dr. Reginald R., Dept. of Anthropology, Harvard University, Peabody Museum, Cambridge 38, Massachusetts

Geiser, Dr. S. W., Southern Methodist University, Dallas, Texas

Gerard, Prof. R. W., Illinois Neuropsychiatric Institute, Chicago 12, Illinois

Gilman, Dr. Lauren C., Dept. of Zoology, University of Miami, Coral Gables, Florida

Goodchild, Dr. Chauncey G., Dept. of Biology, Emory University, Emory University, Georgia

Goodrich, Dr. H. B., Wesleyan University, Middletown, Connecticut

Gottschall, Dr. Gertrude Y., 315 E. 68th Street, New York 21, New York

Gould, Dr. H. N., Medical Sciences Information Exchange, 1113 Dupont Circle Bldg., Washington, D. C.

Graham, Dr. Herbert, Director, Woods Hole Laboratory, Fish and Wild Life Service, Woods Hole, Massachusetts

Grand, Mr. C. G., Dade County Cancer Institute, 1155 N. W. 15th Street, Miami, Florida

Grant, Dr. M. P., Sarah Lawrence College, Bronxville, New York

Gray, Prof. Irving E., Duke University, Durham, North Carolina

Green, Dr. James W., Dept. of Physiology, Rutgers University, New Brunswick, New Jersey

Gregory, Dr. Louise H., 1160 Fifth Avenue, New York City, New York

Gregg, Dr. James H., University of Florida, Gainesville, Florida

Gregg, Dr. J. P., Dept. of Zoology, Columbia University, New York 27, New York

Grosch, Dr. Daniel S., Dept. of Zoology, North Carolina State College, Raleigh, North Carolina

Grundfest, Dr. Harry, College of Physicians and Surgeons, Columbia University, New York City

Gudernatsch, Dr. Frederick, 41 Fifth Avenue, New York 3, New York
Guthrie, Dr. Mary J., Detroit Institute for Cancer Research, 4811 John R Street, Detroit 1, Michigan
Guttman, Dr. Rita, Dept. of Physiology, Brooklyn College, Brooklyn, New York
Guyer, Prof. Michael F., University of Wisconsin, Madison, Wisconsin
Hague, Dr. Florence, Sweet Briar College, Sweet Briar, Virginia
Hajdu, Dr. Stephen, U. S. Public Health Institute, Bethesda, Maryland
Hall, Prof. Frank G., Duke University, Durham, North Carolina
Hamburger, Dr. Viktor, Dept. of Zoology, Washington University, St. Louis, Missouri
Hamilton, Dr. Howard L., Iowa State College, Ames, Iowa
Hance, Dr. Robert T., Box 108, R. R. Number 3, Loveland, Ohio
Harman, Dr. Mary T., Kansas State Agricultural College, Manhattan, Kansas
Harnly, Dr. Morris H., Washington Square College, New York University, New York City, New York
Harrison, Prof. Ross G., Yale University, New Haven, Connecticut
Hartline, Dr. H. Keffer, 215 Mergenthaler Hall, Johns Hopkins University, Baltimore 18, Maryland
Hartman, Dr. Frank A., Hamilton Hall, Ohio State University, Columbus, Ohio
Harvey, Dr. Ethel Browne, 48 Cleveland Lane, Princeton, New Jersey
Harvey, Dr. E. Newton, Guyot Hall, Princeton University, Princeton, New Jersey
Hauschka, Dr. T. S., Institute for Cancer Research, Experimental Zoology, Fox Chase, Philadelphia 11, Pennsylvania
Haxo, Dr. Francis T., Division of Marine Botany, Scripps Institution of Oceanography, University of California, La Jolla, California
Hayashi, Dr. Teru, Dept. of Zoology, Columbia University, New York City, New York
Hayden, Dr. Margaret A., 34 Weston Road, Wellesley 81, Massachusetts
Hayes, Dr. Frederick R., Zoology Laboratory, Dalhouse University, Halifax, Nova Scotia
Haywood, Dr. Charlotte, Mount Holyoke College, South Hadley, Massachusetts
Heilbrunn, Dr. L. V., Dept. of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania
Hendley, Dr. Charles D., 281 Fir Street, Park Forest, Illinois
Henley, Dr. Catherine, Dept. of Zoology, University of North Carolina, Chapel Hill, North Carolina
Henshaw, Dr. Paul S., 17th Floor, 501 Madison Avenue, New York 22, New York
Hess, Prof. Walter N., Hamilton College, Clinton, New York
Hibbard, Dr. Hope, Dept. of Zoology, Oberlin College, Oberlin, Ohio
Hill, Dr. Samuel E., 135 Brunswick Road, Troy, New York
Hinrichs, Dr. Marie, Board of Education, Bureau of Health Service, 228 N. LaSalle Street, Chicago, Illinois
Hisaw, Dr. F. L., Harvard University, Cambridge, Massachusetts
Hoadley, Dr. Leigh, Harvard University Biological Laboratories, Cambridge, Massachusetts

Hodes, Dr. Robert, Nuffield Dept. of Orthopaedic Surgery, Oxford, England
Hodge, Dr. Charles, IV, Temple University, Dept. of Zoology, Philadelphia, Pennsylvania
Hogue, Dr. Mary J., University of Pennsylvania Medical School, Philadelphia, Pennsylvania
Hollaender, Dr. Alexander, P. O. Box W, Clinton Laboratories, Oak Ridge, Tennessee
Hopkins, Dr. Dwight L., University of Illinois, Pier Branch, Navy Pier, Division of Biological Sciences, Chicago, Illinois
Hopkins, Dr. Hoyt S., New York University College of Dentistry, New York City, New York
Hunter, Dr. Francis R., Institute for T. B. Research, Rm. 201, 1835 W. Harrison Street, Chicago 12, Illinois
Hutchens, Dr. John O., Dept. of Physiology, University of Chicago, Chicago 37, Illinois
Hyman, Dr. Libbie H., American Museum of Natural History, New York City, New York
Irving, Dr. Laurence, U. S. Public Health Service, Anchorage, Alaska
Iselin, Mr. Columbus O'D., Woods Hole, Massachusetts
Jacobs, Prof. M. H., School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania
Jenkins, Dr. George B., 5339 42 Street, N. W., Washington 15, D. C.
Jenner, Dr. Charles E., Dept. of Zoology, University of North Carolina, Chapel Hill, North Carolina
Jones, Dr. E. Ruffin, Jr., Biology Department, University of Florida, Gainesville, Florida
Kaan, Dr. Helen W., National Heart Institute, National Institute of Health, Bethesda 14, Maryland
Kabat, Dr. E. A., Neurological Institute, College of Physicians and Surgeons, New York City, New York
Karush, Dr. Fred, Dept. of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania
Kaufman, Prof. B. P., Carnegie Institute, Cold Spring Harbor, Long Island, New York
Kempton, Prof. Rudolf T., Vassar College, Poughkeepsie, New York
Ketchum, Dr. Bostwick, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
Kille, Dr. Frank R., Carleton College, Northfield, Minnesota
Kind, Dr. C. Albert, Dept. of Chemistry, University of Connecticut, Storrs, Connecticut
Kindred, Dr. J. E., University of Virginia, Charlottesville, Virginia
King, Dr. Robert L., State University of Iowa, Iowa City, Iowa
Kisch, Dr. Bruno, 845 West End Avenue, New York City, New York
Kleinholz, Dr. Lewis H., Department of Biology, Reed College, Portland, Oregon
Klotz, Dr. I. M., Department of Chemistry, Northwestern University, Evanston, Illinois

Knowlton, Prof. F. P., 1356 Westmoreland Avenue, Syracuse, New York

Kopac, Dr. M. J., New York University, Washington Square College, New York City, New York

Korr, Dr. I. M., Dept. of Physiology, Kirksville College of Osteopathy and Surgery, Kirksville, Missouri

Krahl, Dr. M. E., Dept. of Physiology, The University of Chicago, Chicago 37, Illinois

Kreig, Dr. Wendall J. S., 303 East Chicago Avenue, Chicago, Illinois

Kunitz, Dr. Moses, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Kuffler, Dr. Stephen, Dept. of Ophthalmology, Johns Hopkins Hospital, Baltimore 5, Maryland

Lackey, Dr. James B., University of Florida, College of Engineering, Gainesville, Florida

Lancefield, Dr. D. E., Queens College, Flushing, New York

Lancefield, Dr. Rebecca C., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Landis, Dr. E. M., Harvard Medical School, Boston 15, Massachusetts

Lange, Dr. Mathilda M., Box 307, Central Valley, New York

Lansing, Dr. Albert I., Dept. of Anatomy, Emory University, Emory, Georgia

Lauffer, Dr. Max A., Dept. of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania

Lavin, Dr. George I., 3714 Springdale Avenue, Baltimore, Maryland

Lazarow, Dr. Arnold, Dept. of Anatomy, University of Minnesota, The Medical School, Minneapolis, Minnesota

Lee, Dr. Richard E., Cornell University College of Medicine, New York City, New York

LeFevre, Dr. Paul G., Div. of Biology and Medicine, U. S. Atomic Energy Commission, Washington 25, D. C.

Lessler, Dr. Milton A., Dept. of Physiology, Ohio State University, Columbus, Ohio

Levy, Dr. Milton, Chemistry Dept., New York University School of Medicine, New York City, New York

Lewis, Prof. I. F., 1110 Rugby Road, Charlottesville, Virginia

Little, Dr. E. P., Wayne University, Detroit, Michigan

Lochhead, Dr. John H., Dept. of Zoology, University of Vermont, Burlington, Vermont

Loeb, Prof. Leo, 40 Crestwood Drive, St. Louis 5, Missouri

Loeb, Dr. R. F., 180 Fort Washington Avenue, New York City, New York

Loewi, Dr. Otto, 155 East 93rd Street, New York City, New York

Lorand, Dr. Laszlo, Wayne University College of Medicine, Detroit, Michigan

Love, Dr. Lois H., 4253 Regent Street, Philadelphia 4, Pennsylvania

Love, Dr. Warner E., Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

Lynch, Dr. Clara J., Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Lynch, Dr. Ruth Stocking, Dept. of Botany, University of California, Los Angeles 24, California

Lynn, Dr. William C., Dept. of Biology, Catholic University of America, Washington, D. C.

MacDougal, Dr. Mary S., Mt. Vernon Apts., 423 Clairmont Avenue, Decatur, Georgia

McCouch, Dr. Margaret Sumwalt, University of Pennsylvania Medical School, Philadelphia, Pennsylvania

McDonald, Sr. Elizabeth Seton, Dept. of Biology, College of Mt. St. Joseph, Mt. St. Joseph, Ohio

McDonald, Dr. Margaret H., Carnegie Institute of Washington, Cold Spring Harbor, Long Island, New York

McGregor, Dr. J. H., Columbia University, New York City, New York

Macklin, Dr. Charles C., Dept. of Anatomy, University of Toronto, Toronto, Canada

Magruder, Dr. Samuel R., Dept. of Anatomy, Tufts Medical School, 136 Harrison Avenue, Boston, Massachusetts

Manwell, Dr. Reginald D., Syracuse University, Syracuse, New York

Marshak, Dr. Alfred, Woods Hole, Massachusetts

Marsland, Dr. Douglas A., New York University, Washington Square College, New York City, New York

Martin, Prof. Earl A., Department of Biology, Brooklyn College, Brooklyn, New York

Mathews, Prof. A. P., Woods Hole, Massachusetts

Matthews, Dr. Samuel A., Thompson Biological Laboratory, Williams College, Williamstown, Massachusetts

Mavor, Prof. James W., 8 Gracewood Park, Cambridge 58, Massachusetts

Mazia, Dr. Daniel, University of California, Department of Zoology, Berkeley 4, California

Medes, Dr. Grace, Lankenau Research Institute, Philadelphia, Pennsylvania

Meigs, Mrs. E. B., 1736 M Street, N. W., Washington, D. C.

Meinkoth, Dr. Norman A., Dept. of Biology, Swarthmore College, Swarthmore, Pennsylvania

Memhard, Mr. A. R., Riverside, Connecticut

Menkin, Dr. Valy, Dept. of Surgical Research, Temple University Medical School, Philadelphia, Pennsylvania

Metz, Dr. C. B., Dept. of Zoology, Florida State University, Tallahassee, Florida

Metz, Prof. Charles W., University of Pennsylvania, Philadelphia, Pennsylvania

Miller, Dr. J. A., Basic Science Building, Emory University, Georgia

Milne, Dr. Lorus J., Department of Zoology, University of New Hampshire, Durham, New Hampshire

Minnich, Prof. D. E., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Mitchel, Dr. Philip H., 1414 Grove Terrace, Winter Park, Florida

Moe, Mr. Henry A., Secretary General, Guggenheim Memorial Foundation, 551 Fifth Avenue, New York 17, New York

Moore, Dr. Carl R., University of Chicago, Chicago 37, Illinois

Moore, Dr. George M., Dept. of Zoology, University of New Hampshire, Durham, New Hampshire
Moul, Dr. E. T., Dept. of Botany, Rutgers University, New Brunswick, New Jersey
Mountain, Mrs. J. D., 9 Coolidge Avenue, White Plains, New York
Muller, Prof. H. J., Dept. of Zoology, Indiana University, Bloomington, Indiana
Nabrit, Dr. S. M., Atlanta University, Morehouse College, Atlanta, Georgia
Nachmansohn, Dr. David, College of Physicians and Surgeons, New York City, New York
Navez, Dr. Albert E., 206 Churchill's Lane, Milton 86, Massachusetts
Neuberg, Dr. Carl, 11 E. 74th Street, New York 21, New York
Neurath, Dr. H., Dept. of Biochemistry, University of Washington, Seattle 5, Washington
Newman, Prof. H. H., 173 Devon Drive, Clearwater, Florida
Nicoll, Dr. Paul A., Dept. of Physiology, Indiana University, Bloomington, Indiana
Ochoa, Dr. Severo, New York University College of Medicine, New York 16, New York
Oppenheimer, Dr. Jane M., Dept. of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania
Osburn, Prof. R. C., Botany-Ecology Bldg., Ohio State University, Columbus 10, Ohio
Oster, Dr. Robert H., University of Maryland, School of Medicine, Baltimore 1, Maryland
Osterhout, Prof. W. J. V., Rockefeller Institute, 66th Street and York Avenue, New York 21
Osterhout, Mrs. Marion Irwin, Rockefeller Institute, 66th Street and York Avenue, New York 21
Packard, Dr. Charles, Woods Hole, Massachusetts
Page, Dr. Irvine H., Cleveland Clinic, Cleveland, Ohio
Pappenheimer, Dr. A. M., 45 Holden Street, Cambridge, Massachusetts
Parmenter, Dr. Charles L., Dept. of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania
Parpart, Dr. Arthur K., Dept. of Biology, Princeton University, Princeton, New Jersey
Passano, Dr. Leonard M., Dept. of Zoology, University of Washington, Seattle, Washington
Patten, Dr. Bradley M., University of Michigan Medical School, Ann Arbor, Michigan
Peebles, Prof. Florence, 380 Rosemont Avenue, Pasadena 3, California
Perkins, Dr. John F., Jr., Dept. of Physiology, University of Chicago, Chicago 37, Illinois
Pettibone, Dr. Marian H., Dept. of Zoology, University of New Hampshire, Durham, New Hampshire
Pierce, Dr. Madelene E., Vassar College, Poughkeepsie, New York
Plough, Prof. Harold H., Amherst College, Amherst, Massachusetts
Pollister, Dr. A. W., Columbia University, New York City, New York

Pond, Dr. Samuel E., 53 Alexander Street, Manchester, Connecticut
Pratt, Dr. Frederick H., 105 Hundreds Road, Wellesley Hills 82, Massachusetts
Proctor, Dr. Nathaniel, Dept. of Biology, Morgan State College, Baltimore 12, Maryland
Prosser, Dr. C. Ladd, 401 Natural History Bldg., University of Illinois, Urbana, Illinois
Provasoli, Dr. Luigi, Dept. of Biology, Haskins Laboratory, 305 E. 43rd Street, New York 17, New York
Quastel, Dr. Juda H., Dept. of Biochemistry, McGill University, Montreal, Canada
Ramsey, Dr. Robert W., Medical College of Virginia, Richmond, Virginia
Rand, Dr. Herbert W., 7 Siders Pond Road, Falmouth, Massachusetts
Rankin, Dr. John S., Dept. of Zoology, University of Connecticut, Storrs, Connecticut
Ratner, Dr. Sarah, The Public Health Research Institute of the City of New York, Foot of East 15th Street, New York 9, New York
Redfield, Dr. Alfred C., Woods Hole, Massachusetts
Reid, Dr. W. M. Monmouth College, Monmouth, Illinois
Reiner, Dr. J. M., Columbia-Presbyterian Medical Center, 622 W. 168 Street, New York 32, New York
Renn, Dr. Charles E., 200 Whitehead Hall, Johns Hopkins University, Baltimore 18, Maryland
Reznikoff, Dr. Paul, Cornell University Medical College, 1300 York Avenue, New York City, New York
Rice, Prof. E. L., 2241 Seneca Avenue, Alliance, Ohio
Richards, Prof. A., 2950E Mabel Street, Tucson, Arizona
Richards, Dr. A. Glenn, Entomology Department, University Farm, University of Minnesota, St. Paul, Minnesota
Richards, Dr. Oscar W., American Optical Company, Research Center, Southbridge, Massachusetts
Rieser, Dr. Peter, Dept. of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania
Rogick, Dr. Mary D., College of New Rochelle, New Rochelle, New York
Romer, Dr. Alfred S., Harvard University, Museum of Comparative Zoology, Cambridge, Massachusetts
Ronkin, Dr. Raphael R., Dept. of Physiology, University of Delaware, Newark, Delaware
Root, Dr. R. W., Dept. of Biology, College of the City of New York, New York City, New York
Root, Dr. W. S., Columbia University College of Physicians and Surgeons, Department of Physiology, New York City, New York
Rose, Dr. S. Meryl, Dept. of Zoology, University of Illinois, Champaign, Illinois
Rosenthal, Dr. Theodore B., Dept. of Anatomy, Indiana University School of Medicine, Bloomington, Indiana
Rossie, Dr. Harold H., Dept. of Radiology, Columbia University, New York 32, New York
Roth, Dr. Jay S., Dept. of Biochemistry, Hahnemann Medical College, Philadelphia 2, Pennsylvania

Rothenberg, Dr. M. A., Chief, Chemical Division, Duguay Proving Ground, Tooele, Utah

Rugh, Dr. Roberts, Radiological Research Laboratory, College of Physicians and Surgeons, New York City

Runnström, Dr. John, Wenner-Grens Institute, Stockholm, Sweden

Ryan, Dr. Francis J., Columbia University, New York City, New York

Sampson, Dr. Myra M., Smith College, Northampton, Massachusetts

Sandeen, Dr. Muriel I., Dept. of Zoology, Duke University, Durham, North Carolina

Saunders, Mr. Lawrence, R. D. 7, Bryn Mawr, Pennsylvania

Schaeffer, Dr. Asa A., Dept. of Biology, Temple University, Philadelphia, Pennsylvania

Scharrer, Dr. Ernst A., University of Colorado, School of Medicine, Dept. of Anatomy, 4200 E. 9th Avenue, Denver 7, Colorado

Schechter, Dr. Victor, College of the City of New York, New York City, New York

Schmidt, Dr. L. H., Christ Hospital, Cincinnati, Ohio

Schmitt, Prof. Francis O., Dept. of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

Schmitt, Dr. O. H., Dept. of Physics, University of Minnesota, Minneapolis 14, Minnesota

Scholander, Dr. P. F., Woods Hole, Massachusetts

Schotté, Dr. Oscar E., Dept. of Biology, Amherst College, Amherst, Massachusetts

Schrader, Dr. Franz, Dept. of Zoology, Columbia University, New York City, New York

Schrader, Dr. Sally Hughes, Dept. of Zoology, Columbia University, New York City, New York

Schramm, Prof. J. R., University of Pennsylvania, Philadelphia, Pennsylvania

Scott, Dr. Allen C., Colby College, Waterville, Maine

Scott, Sister Florence M., Seton Hill College, Greensburg, Pennsylvania

Scott, Dr. George T., Oberlin College, Oberlin, Ohio

Sears, Dr. Mary, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Semple, Mrs. R. Bowling, 1767 P Street, N.W., Washington, D. C.

Severinghaus, Dr. Aura E., Dept. of Anatomy, College of Physicians and Surgeons, New York City, New York

Shanes, Dr. Abraham M., Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda 14, Maryland

Shapiro, Dr. Herbert, 5800 N. Camac Street, Philadelphia 41, Pennsylvania

Shaver, Dr. John R., Research Fellow, California Institute of Technology, Pasadena, California

Shedlovsky, Dr. Theodore, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Shumway, Dr. Waldo, Stevens Institute of Technology, Hoboken, New Jersey

Sichel, Dr. Ferdinand J. M., University of Vermont, Burlington, Vermont

Sichel, Mrs. F. J. M., 35 Henderson Terrace, Burlington, Vermont

Silva, Dr. Paul, Dept. of Botany, University of Illinois, Urbana, Illinois

Slifer, Dr. Eleanor H., Dept. of Zoology, State University of Iowa, Iowa City, Iowa

Smith, Dr. Dietrich C., Dept. of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

Smith, Dr. Edward H., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Smith, Mr. Homer P., General Manager, Marine Biological Laboratory, Woods Hole, Massachusetts

Smith, Dr. Ralph I., Dept. of Zoology, University of California, Berkeley 4, California

Sonneborn, Dr. T. M., Dept. of Zoology, Indiana University, Bloomington, Indiana

Sonnenblick, Dr. B. P., 40 Rector Street, Newark 2, New Jersey

Speidel, Dr. Carl C., University of Virginia, University, Virginia

Spratt, Dr. Nelson T., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Starr, Dr. Richard C., Dept. of Botany, Indiana University, Bloomington, Indiana

Steinbach, Dr. Henry Burr, 6211 33rd Street, N. W., Washington, D. C.

Stephens, Dr. Grover C., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota

Stern, Dr. Kurt G., Polytechnic Institute, Dept. of Chemistry, 84 Livingston Street, Brooklyn, New York

Stewart, Dr. Dorothy, Rockford College, Rockford, Illinois

Stokey, Dr. Alma G., Dept. of Botany, Mt. Holyoke College, South Hadley, Massachusetts

Straus, Dr. W. L., Johns Hopkins University, Baltimore 18, Maryland

Stunkard, Dr. Horace W., New York University, New York City, New York

Sturtevant, Dr. Alfred H., California Institute of Technology, Pasadena 4, California

Sulkin, Dr. S. Edward, Dept. of Bacteriology, Southwestern Medical School, University of Texas, Dallas, Texas

Swope, Mr. Gerard, Jr., 570 Lexington Avenue, New York 22, New York

Szent-Györgyi, Dr. A. E., Woods Hole, Massachusetts

Szent-Györgyi, Dr. Andrew G., Institute for Muscle Research, Woods Hole, Massachusetts

Tashiro, Dr. Shiro, University of Cincinnati Medical College, Cincinnati, Ohio

Taylor, Dr. Wm. Randolph, University of Michigan, Ann Arbor, Michigan

TeWinkel, Dr. Lois E., Dept. of Zoology, Smith College, Northampton, Massachusetts

Tracy, Dr. Henry C., Dept. of Anatomy, University of Mississippi, University, Mississippi

Trager, Dr. William, Rockefeller Institute, 66th Street and York Avenue, New York 21, New York

Trinkaus, Dr. J. Philip, Dept. of Zoology, Osborn Zoological Laboratory, New Haven, Connecticut

Turner, Prof. C. L., Northwestern University, Evanston, Illinois

Tyler, Dr. Albert, California Institute of Technology, Pasadena, California
Uhlenhuth, Dr. Edward, University of Maryland School of Medicine, Baltimore, Maryland
Villee, Dr. Claude A., Harvard Medical School, Boston 15, Massachusetts
Vincent, Dr. Walter S., Dept. of Anatomy, State University of New York School of Medicine, Syracuse 10, New York
Wainio, Dr. W. W., Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey
Wald, Dr. George, Biological Laboratory, Harvard University, Cambridge 38, Massachusetts
Warbasse, Dr. James P., Woods Hole, Massachusetts
Warner, Dr. Robert C., Dept. of Chemistry, New York University College of Medicine, New York 16, New York
Waterman, Dr. T. H., Osborn Zoological Laboratory, Yale University, New Haven, Connecticut
Webb, Dr. Marguerite, Dept. of Physiology and Bacteriology, Goucher College, Towson, Maryland
Weiss, Dr. Paul A., Dept of Zoology, University of Chicago, Chicago 37, Illinois
Wenrich, Dr. D. H., University of Pennsylvania, Philadelphia, Pennsylvania
Whedon, Dr. A. D., 21 Lawncrest, Danbury, Connecticut
Whitaker, Dr. Douglas M., P. O. Box 2514, Stanford University, California
White, Dr. E. Grace, Wilson College, Chambersburg, Pennsylvania
Whiting, Dr. Anna R., University of Pennsylvania, Philadelphia, Pennsylvania
Whiting, Dr. Phineas W., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania
Wickersham, Mr. James H., 530 Fifth Avenue, New York 36, New York
Wichterman, Dr. Ralph, Biology Dept., Temple University, Philadelphia, Pennsylvania
Wieman, Prof. H. L., University of Cincinnati, Cincinnati, Ohio
Wilber, Dr. C. G., Medical Laboratories, Applied Physiology Branch, Army Chemical Center, Maryland
Willier, Dr. B. H., Dept. of Biology, Johns Hopkins University, Baltimore, Maryland
Wilson, Dr. J. W., Brown University, Providence, Rhode Island
Wilson, Dr. Walter L., Dept. of Physiology, University of Vermont College of Medicine, Burlington, Vermont
Witschi, Prof. Emil, Dept. of Zoology, State University of Iowa, Iowa City, Iowa
Wolf, Dr. Ernst, Pendleton Hall, Wellesley College, Wellesley, Massachusetts
Woodward, Dr. Alvalyn E., Route Number 1, Bay View, Mt. Pleasant, South Carolina
Woodward, Dr. Arthur A., Army Medical Center, Maryland (Applied Physiology Branch, Army Chemical Corps, Medical Laboratory)
Wright, Dr. Paul A., Dept. of Zoology, University of Michigan, Ann Arbor, Michigan
Wrinch, Dr. Dorothy, Dept. of Physics, Smith College, Northampton, Massachusetts

YNTEMA, DR. C. L., Dept. of Anatomy, State University of New York, College of Medicine, Syracuse 10, New York

YOUNG, DR. D. B., Main Street, North Hanover, Massachusetts

ZINN, DR. DONALD J., Zoology Dept., University of Rhode Island, Kingston, Rhode Island

ZORZOLI, DR. ANITA, Dept. of Physiology, Southern Illinois University, Carbondale, Illinois

ZWILLING, DR. E., Dept. of Genetics, University of Connecticut, Storrs, Connecticut

3. ASSOCIATE MEMBERS, 1954

ALDRICH, AMY OWEN
ALTON, DR. BENJAMIN H.
ANTHONY, MR. RICHARD A.
ARMSTRONG, MRS. LOUISE D.
ARMSTRONG, DR. AND MRS. P. B.
BAITSELL, DR. AND MRS. GEORGE A.
BARBOUR, MR. LUCIUS
BARTOW, MR. AND MRS. CLARENCE
BARTOW, MRS. FRANCIS D.
BARTOW, MRS. PHILIP
BELL, MARION FENNO
BRADLEY, MR. ALBERT L.
BRADLEY, MR. MAYNARD RIGGS
BROWN, SARAH MEIGS
BURDICK, MR. CHARLES L.
CAHOON, MRS. SAMUEL
CALKINS, MR. G. NATHAN, JR.
CALKINS, MRS. GARY N.
CALKINS, MR. SAMUEL
CARLETON, MARGARET D.
CLAFF, MR. AND MRS. C. LLOYD
CLARK, DR. AND MRS. ALFRED HULL
CLARK, MRS. LEROY
CLARK, MR. W. VAN ALAN
CLOWES, MR. ALLEN W.
CLOWES, MRS. G. H. A.
CLOWES, DR. AND MRS. GEORGE, JR.
COLTON, MR. H. SEYMOUR
CRANE, MRS. LOUISE
CRANE, MRS. CARY W.
CRANE, MRS. FRANCES A.
CRANE, MRS. W. MURRAY
CROSSLEY, MR. AND MRS. ARCHIBALD M.
CROWELL, MR. PRINCE S.
DANIELS, MR. AND MRS. F. HAROLD
DAY, MR. AND MRS. POMEROY
DRAPER, MRS. MARY C.
DRINKER, DR. AND MRS. CECIL K.
ELSMITH, MRS. DOROTHY
ENDRES, MR. FRED
FAY, MR. AND MRS. HENRY H.
FISHER, MRS. BRUCE CRANE
FROST, MR. EUGENE M.
GALTSOFF, DR. PAUL S.
GARFIELD, MRS. I. McD.
GIFFORD, MRS. BARBARA
GIFFORD, MR. AND MRS. JOHN A.
GILCHRIST, MR. AND MRS. JOHN A.
GILDEA, DR. AND MRS. E. F.
GREEN, MISS GLADYS W.
HAMLEN, MR. J. MONROE
HARRELL, MR. AND MRS. JOEL E.
HARRINGTON, MR. ROBERT D.
HOUSTON, MR. AND MRS. HOWARD E.
HOWE, MRS. HARRISON E.
JANNEY, MRS. WALTER C.
JEWETT, MR. AND MRS. GEORGE F.
KEITH, MR. HAROLD C.
KIDDER, MRS. HENRY M.
KING, MR. FRANKLIN
KOLLER, MRS. LEWIS
LAWRENCE, MR. MILFORD
LEMANN, MRS. SOLEN B.
LILLIE, MRS. FRANK R.
LILLIE, MRS. RALPH S.
LOBB, MRS. JOHN
McCLINTIC, MRS. GUTHRIE
McLANE, MR. T. THORNE
MARVIN, MRS. WALTER T.
MAST, GRACE T.
MEIGS, MRS. EDWARD B.
MEIGS, MRS. J. WISTER
MEIGS, MRS. J. WISTER, JR.
MELLON, MRS. RICHARD K.

Miskell, Mr. Joseph B.
Mitchell, Mrs. James McC.
Mixter, Mrs. Jason
Montgomery, Mrs. T. H.
Moore, Miss Mary Louise G.
Moore, Mrs. William A.
Mosser, Mrs. Florence M.
Motley, Mrs. Thomas
Newton, Miss Helen K.
Nichols, Mrs. George
Nims, Mrs. E. D.
Norman, Mr. Edward A.
Packard, Dr. and Mrs. Charles
Park, Mr. Malcolm S.
Peck, Mr. and Mrs. Samuel A.
Pennington, Miss Anne H.
Redfield, Mrs. Alfred
Reznihoff, Dr. Paul
Riggs, Mrs. Lawrason
Rivinus, Anne H.
Rhodes, Mrs. Boyle
Root, Mrs. Walter
Rudd, Mrs. H. W. Dwight
Sands, Miss Adelaide G.
Saunders, Mrs. Lawrence
Sinclair, Mr. W. R.
Smith, Mrs. Edward H.
Stanwood, Mrs. F. A.
Stone, Tilda Baer
Swift, Mr. and Mrs. E. Kent
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Tilney, Mrs. Augusta M.
Tompkins, Mr. and Mrs. B. A.
Vanneman, Dr. and Mrs. Joseph
Warbasse, Dr. James P.
Webster, Mrs. Edwin S.
Whitely, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Williston, Miss Emily
Williston, Prof. Samuel
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Sarah A.

V. REPORT OF THE LIBRARIAN

1954

In 1954, the number of currently-received journals totalled 1523 (50 new). Of these titles, there were 460 (9 new) Marine Biological Laboratory subscriptions; 607 (16 new) exchanges and 169 (10 new) gifts; 77 (4 new) were Woods Hole Oceanographic Institution subscriptions; 171 (5 new) were exchanges and 39 (6 new) were gifts.

The Marine Biological Laboratory purchased 42 books, received 63 complimentary copies (5 from authors and 58 from publishers) and 53 miscellaneous presentations. The Woods Hole Oceanographic Institution purchased 10 titles and received 5 gifts. The total number of books accessioned was 173.

The Marine Biological Laboratory completed by purchase 4 journal sets and partially completed 12 sets. The Woods Hole Oceanographic Institution completed 3 sets and partially completed 2 sets. Volumes and numbers received by gift and by exchange completed 5 sets and partially completed 9 sets.

The reprint collection gained 3614 papers of which 2168 were of current issue.

Forty-nine items were borrowed on inter-library loan, and 165 were sent to out-of-town libraries. The sale of duplicate material contributed $259.40 to the Laboratory's General Fund.

During the year, several very fine gifts were presented to the Library. Dr. R. P. Bigelow, continuing his keen interest in the Library, presented 8 books among which were several valuable scientific classics. To Dr. Bruno Kisch, grateful acknowledgment is made of the very fine etching of Prof. L. Agassiz made shortly after his death by the artist Anna Merritt.

Other gifts included 13 books contributed by Dr. Henry Stommel, 6 by Dr. Dorothy Wrinch, and 6 by Dr. Richard Bliss. A total of 266 reprints were received from Drs. A. C. Redfield, A. Lazarow and P. W. Whiting.

The California Institute of Technology Library, at the suggestion of Dr. A. H. Sturtevant, returned to the Laboratory a valuable run of the "Biological Bulletin." This gift provided the opportunity to replenish stock which is exceedingly low.

In September, an Assistant Librarian was appointed, thus filling a position which has been vacant since 1947. The Library Committee and the Librarian have for a long time been fully aware of the need of an assistant, and it is a satisfaction to have this position re-established.

At the end of the year, the Library contained 64,272 bound volumes and 192,198 reprints.

Respectfully submitted,

Deborah L. Harlow
Librarian

VI. REPORT OF THE TREASURER

A summary of the securities held in the Endowment Fund appears in the fifth exhibit. On January 1, 1955, these securities had a market value of $1,359,041 compared with $1,153,996 on January 1, 1954. The average yield on the securities was 4.87% on the book value and 3.5% on the market value.

Exhibit 1 is the report of our new auditors, Lybrand, Ross Bros. & Montgomery of Boston on the examination of our financial statements.

The second exhibit gives the Balance Sheet. It reflects the situation as of December 31, 1954, of Bar Neck Property. At that time negotiations were still being conducted for a more equitable settlement than the original amount of $75,000 fixed by the U. S. Navy. Since the first of the year an agreement was reached in the amount of $115,000 and payment has been received of the balance. From this total must be deducted expenses (legal, appraisals, etc.) amounting to $10,328.44. The Laboratory thus has received $104,671.56 net for the property.

The third exhibit is a condensed statement of operating expenditures and income. It should be pointed out that as of December 31, 1954, the Laboratory had spent $21,263.54 towards the repairs made necessary by Hurricane Carol. This expenditure is included in the statement. Since the close of the year a grant in the amount of $50,000 was received from the National Science Foundation in response to a Laboratory application for $61,500 as the total extent of our losses. Inasmuch as the rehabilitation program extended into the year 1955, additional expenditures will be reflected in the report of next year.

Again I would like to remind all who are concerned with the Laboratory of the need for their continued efforts to develop new sources of income. To an ever increasing extent the success of the Laboratory program is directly proportional to our fund raising abilities. A great deal of effort has recently gone into a brochure that is now available to describe the varied activities of the Laboratory.

Your officers and the members of the Executive Committee feel that this will be very useful to all of us in seeking contributions from individuals, foundations, and corporations which should be interested in supporting the basic research and teaching being done at the Marine Biological Laboratory.

Exhibit 1

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts.

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1954 and the related statements of operating expenditures and income and current fund for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1954 and the expenditures and income for the year then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts
July 15, 1955

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

Exhibit 2

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1954

Investments

Investments held by Trustee:		
Securities, at cost (approximate market quotation $1,359,041)		$ 979,104
Cash		1,428
		980,532
Investments of other endowment funds:		
Securities, at cost (approximate market quotation $25,255)	$19,436	
Cash	8,227	27,663
Investments of unrestricted funds		198,710
		1,206,905

Plant Assets

Land, buildings, library and equipment	2,392,533
Less allowance for depreciation (note A)	921,483
	1,471,050

Current Assets

Cash	21,128
Accounts receivable ($13,441 from U. S. Government)	30,570
Inventories of specimens and bulletins	58,341
Prepaid insurance and other	8,359
	118,398
	$2,796,353

Note A—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

Exhibit 2,
continued

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1954

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 980,532
Endowment funds for awards and scholarships		
Principal	$ 18,156	
Unexpended income	9,507	27,663
Unrestricted funds functioning as endowment		198,710
		1,206,905

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant	2,387,533	
Less allowance for depreciation charged thereto	921,483	1,466,050
		1,471,050

Current Liabilities and Fund

Accounts payable	31,275
Unexpended balances of gifts for designated purposes	2,222
Unexpended research grant	315
Current fund	84,586
	118,398
	$2,796,353

Exhibit 3

Marine Biological Laboratory

Statement of Operating Expenditures and Income

Year Ended December 31, 1954

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$150,999
Instruction	25,161
Library, including book purchases	25,373
Biological Bulletin	15,035
	216,568
Administration and general	42,294
Plant operation and maintenance	59,793
Hurricane emergency repairs	21,264
Dormitories and dining services	114,756
Equipment purchased from current funds	7,433
	462,108
Less depreciation included in plant operation and auxiliary activities above but charged to plant funds	36,171
	425,937

Income

Direct income of departments:		
Research fees		41,927
Accessory services:		
Sales of biological specimens	$66,619	
Other credits for accessory services	27,403	94,022
Instruction fees		16,345
Library fees and income		6,869
Biological Bulletin, subscriptions and sales		16,017
		175,180
Allowance for indirect costs on research contracts		13,765
Dormitories and dining services income		92,569
		281,514
Investment income:		
General endowment	47,725	
Current fund investments	22,851	70,576
Gifts for current use		50,586
Sundry income		3,062
Total current income		405,738
Excess of operating expenditures over income		$ 20,199

Direct costs of $36,163 on research contracts and reimbursement therefor are not included in operating expenditures or income.

Exhibit 4

MARINE BIOLOGICAL LABORATORY

STATEMENT OF CURRENT FUND

Year Ended December 31, 1954

Balance January 1, 1954		$249,043
Less:		
Adjustment of credit to account in 1953 for taking of Bar Neck Property	$ 19,536	
Amount transferred to unrestricted funds functioning as endowment as of December 31, 1954 pursuant to vote of Executive Committee of the Board of Trustees	124,722	144,258
		104,785
Excess of operating expenditures over income, 1954		20,199
Balance December 31, 1954		$ 84,586

Exhibit 5

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1954

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1954
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$185,244	22.5	$ 184,736	16.5	$ 5,200
Other bonds	285,747	34.8	293,654	26.2	7,902
	470,991	57.3	478,390	42.7	13,102
Preferred stocks	85,788	10.5	80,856	7.2	3,614
Common stocks	264,150	32.2	562,996	50.1	22,800
	$820,929	100.0	$1,122,242	100.0	$39,516
General Educ. board endow. fund:					
U. S. Government bonds	48,161	30.5	48,235	20.4	1,125
Other bonds	30,690	19.4	31,050	13.1	998
	78,851	49.9	79,285	33.5	2,123
Preferred stocks	27,281	17.2	26,485	11.2	1,130
Common stocks	52,043	32.9	131,029	55.2	4,956
	158,175	100.0	236,799	100.0	8,209
Total securities held by trustee	$979,104		$1,359,041		$47,725

Exhibit 5,
continued

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1954

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1954
Securities of other endow. funds:					
Rev. A. Boyer scholar. fund:					
U. S. Government bonds........	5,504	100.0	5,385	100.0	148
The Bio Club scholar. fund:					
U. S. Government bonds........	2,062	50.6	1,955	46.7	51
Other bonds..................	2,012	49.4	2,234	53.3	82
	4,074	100.0	4,189	100.0	133
Allen R. Memhard Fund:					
U. S. Government bonds........	1,000	100.0	978	100.0	28
Lucretia Crocker Fund:					
U. S. Government bonds........	4,515	51.0	4,425	30.0	114
Other bonds..................	4,343	49.0	10,278	70.0	396
	8,858	100.0	14,703	100.0	510
Total securities of other endowment funds..	$ 19,436		$ 25,255		$ 819
Investments of unrestricted funds:					
U. S. Government bonds............	27,300	18.4	26,818	17.1	961
Other bonds......................	68,249	46.1	70,826	45.0	1,228
	95,549	64.5	97,644	62.1	2,189
Common stocks...................	52,481	35.5	59,513	37.9	2,882
	148,030	100.0	$ 157,157	100.0	5,071
Investment in Gen. Biol. Supply House, Inc.........................	12,700				17,780
	160,730				22,851
Real estate and mortgage..........	37,980				
Total investments of unrestricted funds....	$198,710				$22,851

PHYSICAL AND BIOLOGICAL PROCESSES DETERMINING THE DISTRIBUTION OF ZOOPLANKTON IN A TIDAL ESTUARY[1]

JOHN P. BARLOW[2]

Woods Hole Oceanographic Institution, Woods Hole, Mass.

Plankton are at the mercy of the water movements that carry them about. These water movements also determine their physical and biological environments. As a result the study of the geographical distribution of plankton has an unique simplicity. The maintenance of an endemic plankton population and its distribution from its endemic center depends primarily on the dynamic balance between two processes: the translocation and dispersion of the population by the water movements, and the reaction of the population to the changes in environment caused by the water movements (Redfield, 1939, 1941; Redfield and Beale, 1940).

In spite of this simplicity, it is difficult in most environments to separate the effects of dispersal by the water movements on their distribution from the effects of reproduction and mortality of the organisms. The physical and analytical difficulties met in understanding and measuring the effects of these processes are, however, lessened by the characteristics of many tidal estuaries.

Estuaries are environments for which the land provides well defined boundaries. These boundaries permit a useful simplification in that the three dimensions in which events take place in the open sea can often be reduced to the two dimensions of a vertical plane in the land-sea axis of the estuary. Moreover, the rivers that drain into estuaries supply fresh water in a continuous and measurable fashion. This fresh water can become a useful tracer by which the rate of circulation of both fresh and salt water can be measured. The recent studies of the circulation in estuaries by Tully (1949), Ketchum (1951a, 1951b), Ketchum, Redfield and Ayers (1951), and Ketchum and Keen (1953) have given realistic descriptions of the circulation based on the behavior of this fresh water.

These studies show that there must be a net seaward movement of mixed salt and fresh water through an estuary adequate to remove the fresh water added by the river. This seaward movement must tend to transport the weakly swimming planktonic organisms. Ketchum (1954) has shown that the rate of this circulation determines the rate at which a plankton population must reproduce in order to maintain itself in an estuary. He has pointed out that the vigor of the circulation may thus limit the kind of plankton that is able to maintain an endemic population in an estuary. Bousfield (in press) has shown that in the Miramichee estuary the distribution of barnacles is influenced by seaward movements of mixed fresh and salt water and the landward counter current associated with it. Huntsman (1954) has used the estuarine circulation in the Bay of Fundy to explain the distribution of the herring populations there.

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[1] Contribution No. 753 from the Woods Hole Oceanographic Institution.

[2] Present address: Department of Oceanography, A. and M. College of Texas, College Station, Texas.

This net seaward transport through an estuary implies that a population living in any part may be increased by additions from landward as well as decreased by losses to seaward. A knowledge of this circulation permits estimates to be made of the net rate at which a population in any part of the estuary is augmented or reduced by the physical processes. If the population is to be maintained, it must be increased or decreased by growth or mortality to offset the effects of the physical processes. Hence, if the rate of the circulation is taken into account, it is possible to determine the biological processes that underlie the distribution of plankton.

The distribution of zooplankton in a small estuary, Great Pond, near Woods Hole, Mass., has been studied. Rates have been computed at which some typical endemic zooplankton must have grown or died to maintain their distribution in face of the circulation. The rates of reproduction and mortality of these same zooplankton have also been estimated in various parts of the estuary from rates of increase or decrease of portions of the population isolated in bottles. There have thus been two independent estimates of the biological processes that maintain the distribution of these zooplankton. It is possible to give some understanding of both the physical and biological processes that determine the distribution of these zooplankton in Great Pond.

These investigations in Great Pond were suggested by Dr. Alfred C. Redfield. The author owes much to him for his continued encouragement, advice and critical analysis of this work. The author is also indebted to Dr. Bostwick H. Ketchum for his general interest in this problem, and his patient help in understanding the relations between plankton and the circulation in estuaries.

Mr. Edward M. Hulburt supplied much valuable information from his studies on the phytoplankton of the pond and helped greatly in the collection of the zooplankton samples.

METHODS

Zooplankton sampling

Great Pond (Fig. 1) is a small tidal estuary in the northern shore of Vineyard Sound. It is 3,300 meters long, with a maximum width of about 300 meters and a maximum depth of a little over two meters. The topography of its basin divides the pond into the several parts, the arm, narrows, main pond, shallows and entrance, as shown in Figure 1. The stations from which plankton samples were regularly taken are also shown. A full description of the pond has been previously given (Barlow, 1952).

Regular surveys of the plankton of Great Pond were made at 10- to 15-day intervals during the spring and summer of 1950 and at longer intervals during the following winter and spring. All zooplankton samples were taken with the quantitative plankton sampler (Clarke and Bumpus, 1950). Silk nets of No. 10 mesh (nominal aperture 0.158 mm.) were used except for samples taken in February, 1951, when the water was so turbid that it was necessary to use No. 2 mesh nets (nominal aperature 0.366 mm.).

On each survey samples were usually taken from the six stations in the pond and a station in the adjacent part of Vineyard Sound. At all stations a surface

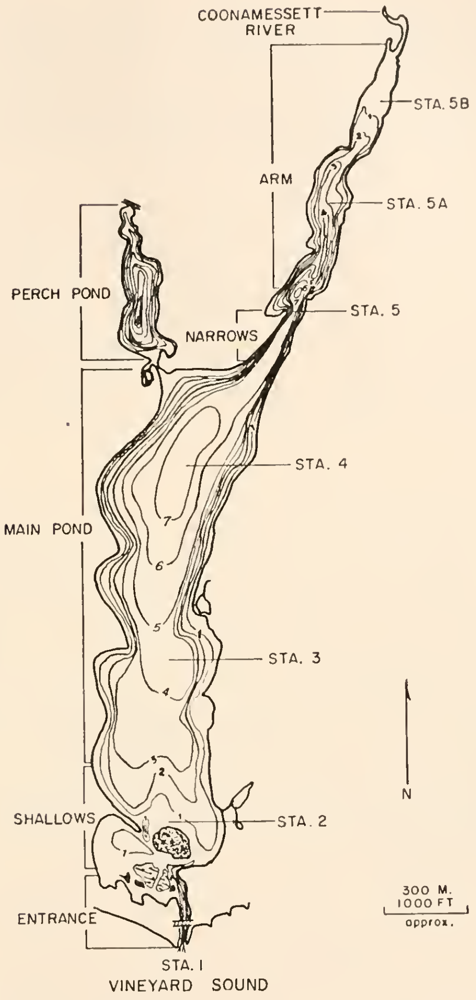
COONAMESSETT RIVER
STA. 5B
ARM
STA. 5A
PERCH POND
STA. 5
NARROWS
STA. 4
MAIN POND
7
6
5
STA. 3
4
3
2
N
SHALLOWS
1
STA. 2
300 M.
1000 FT
approx.
ENTRANCE
STA. 1
VINEYARD SOUND

FIGURE 1. Bathymetric chart of Great Pond; depth in feet.

sample was collected by towing a sampler just below the surface. At stations 4, 5 and 5a, another sample was taken simultaneously as close as possible to the bottom. Tows were made, as far as possible, transverse to the long axis of the pond and at a velocity of two to three knots. The cyclometer readings indicated that usually two to three cubic meters of water were filtered for each sample.

Aliquots of each sample were counted under a dissecting microscope. The size of the aliquots was adjusted so that 50 to 100 of each of the important forms were counted. The concentrations in numbers per cubic meter have been computed from the numbers counted in these aliquots and the volume of water filtered for each sample.

The estimates of abundance of plankton are based on counts of single aliquots from single samples. The representativeness of such counts was tested by duplicate aliquots of a series of ten successive samples taken within two hours at station 3. Analysis for the variance between counts and the variance between hauls by the method of Silliman (1946) indicated that a sample could be considered to be significantly different from another only if it were less than half or more than twice the size of the other.

The samples have usually included so much detritus that the usual direct measurement of the weight or volume of the zooplankton could not be used. Instead, the average dimensions of each organism were determined in several representative samples. Each organism was considered to approximate in shape a simple geometric solid, and its average volume was calculated for each of these representative samples. These calculated average volumes were similar to those arrived at by more elaborate methods by Lohmann (1908) for the same or similar organisms. They have been used to compute the "calculated volume" of zooplankton in each sample.

Experiments with isolated water samples

A number of experiments were made with natural populations of phytoplankton and zooplankton imprisoned in bottles in order to study grazing rates, multiplication and growth. Water was pumped from the desired depth into a large wooden tub. After thorough mixing, one-liter aliquots of this water were poured alternately into a clean five-gallon carboy and through a No. 10 net. When the bottle contained 15 liters it was stoppered and moored where the sample had been taken. Thus filled, the bottle floated with its neck awash, and the water in it was stirred and aerated by wave motion. The sample collected in the net was preserved as a control. Usually bottles were filled and moored at five or six places in the pond. They were harvested after two or three days by pouring their contents through a plankton net. Occasionally some species not usually abundant in the pond would become very abundant in the bottle. Usually, however, the species composition changed very little during the course of an experiment.

As a measure of grazing rate this method was not entirely satisfactory, partly because large changes in the number and sizes of the animals during the course of the experiments made the results difficult to interpret. However, the multiplication and growth of the zooplankton in some of the jugs was so great that some conclusions could be drawn about the response of the animals to the differences in environment in different parts of the pond.

Results

The zooplankton population of Great Pond

There have been a number of studies of the neritic plankton fauna of the Vineyard Sound region. Among these are Fish's (1925) seasonal study of the plankton of Woods Hole harbor and Wilson's (1932) description of the copepods of the Woods Hole region including several smaller estuaries. Deevey (1948) has described a population in Tisbury Great Pond, Martha's Vineyard, that was very similar in composition, seasonal distribution and abundance to that found in the central part of Great Pond.

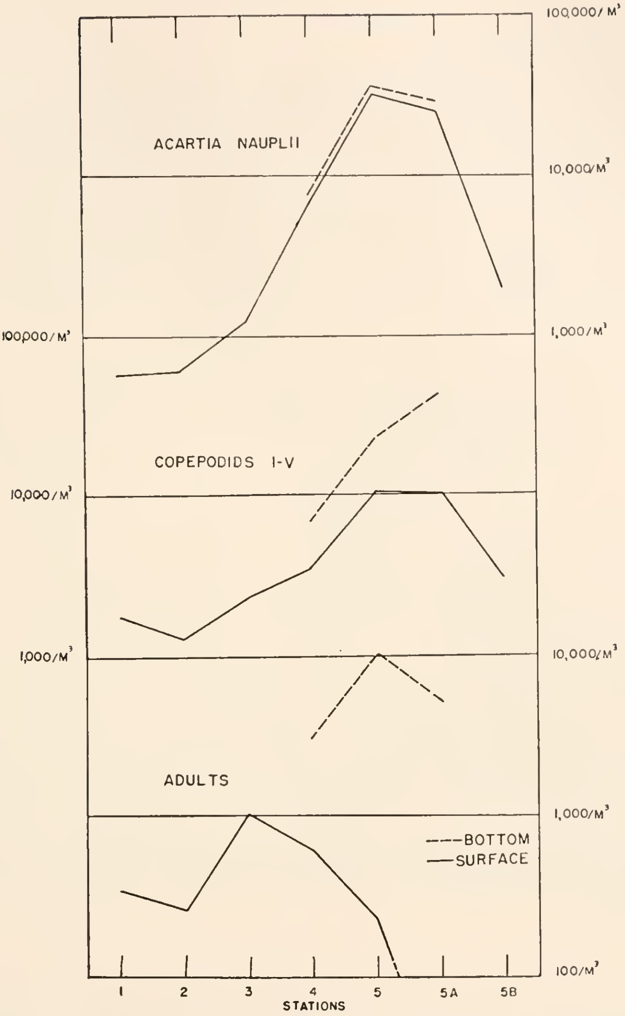

FIGURE 2. Distribution of *Acartia tonsa* copepodids and *Acartia* spp. nauplii in June 1950. Mean of surveys of June 5, 15 and 30.

The principal constituents of the catches from both the Sound and the pond have almost always been small neritic copepods, usually species of *Acartia*. During the late spring and summer these copepods were so much more abundant in parts of the pond than in the Sound that they must have been living endemically somewhere in the pond. The distribution of *Acartia tonsa* copepodid stages and *Acartia sp.* nauplii in the month of June is shown in Figure 2. The species of *Acartia* nauplii has not been determined, but since *A. tonsa* was by far the most abundant *Acartia* in June, it may be presumed that most of the *Acartia* nauplii were of *tonsa*. In addition to these copepods, several other endemic plankton organisms were abundant in this period. The cladoceran *Podon polyphemoides* and species of the rotifer *Synchaeta* were especially numerous in some parts of the pond but were scarce or not found in the Sound.

In contrast there were only a few forms, such as the copepod *Centropages hamatus,* that were more abundant in the Sound than in the pond.

The seasonal change in abundance of plankton has been summarized in Figure 3. This figure shows the seasonal distribution of the calculated volumes of all zooplankton except medusae, ctenophores, and gastropod veligers. The medusae and ctenophores were omitted because of their high water content. The gastropod veligers were omitted because their distribution must at least in part be determined by the distribution of the adult gastropods. Other temporary plankton made but small contributions to the calculated volumes shown in Figure 3.

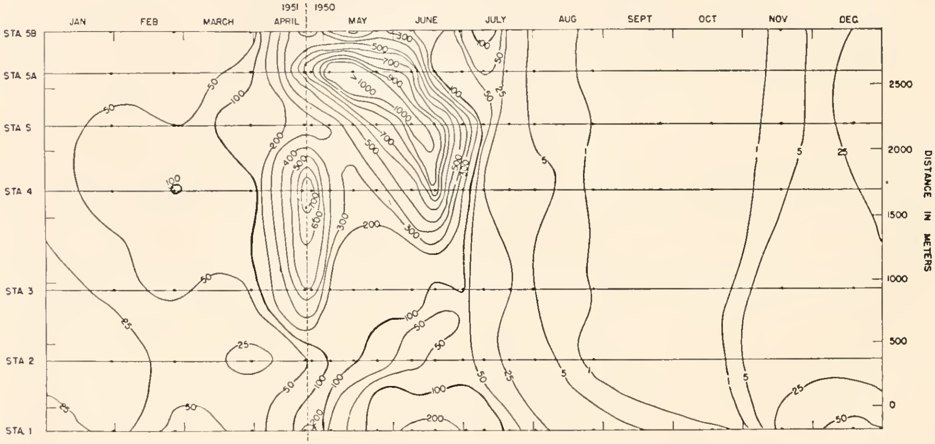

FIGURE 3. Seasonal distribution of zooplankton volume. Calculated volume in mm.^{3}/M^3. Gastropods, hydromedusae, and ctenophores not included. Mean of surface and deeper samples at Stations 4, 5 and 5a. Closed circles indicate observations. Distance in meters from entrance shown at right.

The fall and winter were periods of least abundance in both the pond and the Sound. The population increased from about the first of April through the spring to the period of greatest abundance in the early summer. In the latter part of July in both 1950 and 1951 there was a dramatic decrease in the abundance of all kinds of zooplankton in the pond. There were no obvious changes in physical, chemical or hydrographic conditions accompanying this decrease. In both years it coincided with the appearance of numbers of *Mnemiopsis leidyi.* These ctenophores were so poorly preserved that they could not be counted in the 1950 samples. Examination of some fresh samples in 1951 showed that at Station 5 they increased from less than one per cubic meter in early July to 35 per cubic meter near the end of July.

Since *Mnemiopsis* is known to be a zooplankton predator (Nelson, 1925), it seems possible that the late summer decrease in zooplankton in Great Pond was due to its depredations. Although Deevey (1948) found a similar late summer decrease in Tisbury Great Pond, she found no ctenophores in her samples. The number of zooplankton in Great Pond did not increase substantially again during the remainder of the year.

The greatest concentration of zooplankton in the pond was found on June 5, 1950, in the deeper water at Station 5a where there were 346,000 organisms per cubic meter having a calculated volume of 2,500 mm.^{3} per M^3. The smallest concentrations of plankton in the pond and Sound were found on September 18, 1950, when there were less than 100 organisms per cubic meter, having a calculated volume less than one mm.^{3} per M^3 at Station 5a. Thus there was a several thousand-fold seasonal decrease in both number and volume of zooplankton in the pond.

The concentrations in the Sound have usually been quite different from those in the pond. On June 5 the zooplankton in the Sound was nearly at its maximum, yet it was only 1/72 in numbers and 1/38 in volume of that in the pond. The zooplankton was never as low in the Sound as in the pond. At the time of the September minimum in the pond the zooplankton was about 20 times greater, in both numbers and volume, in the Sound. The winter minimum was about 1/15 the summer maximum in the Sound.

Vertical distribution of plankton in Great Pond

There were usually no significant differences in the concentrations of *Acartia* nauplii at the surface and near the bottom. The juveniles and adult copepodids were, however, found in significantly greater concentrations near the bottom than at the surface at some stations. The difference in surface and bottom concentrations did not appear to be related to the time of day when the samples were taken, as shown by samples taken near midday and midnight of the same day. The distribution of zooplankton does not, therefore, seem to be greatly affected by vertical diurnal migrations. Large differences in surface and bottom concentrations were found when and where there were large differences in surface and bottom salinities. It is probable that the passive sinking of the plankton from the less buoyant brackish surface layer and then active avoidance of this layer are more effective in influencing the concentration of the heavier and more active forms than is a migration influenced by light.

Horizontal distribution of plankton in Great Pond

Tidal exchanges of salt and fresh water play a large part in effecting the salinity distribution in many tidal estuaries. Ketchum (1951b) has given an empirical method for estimating the changes of salt and fresh water in tidal estuaries. This method has been used to estimate the tidal exchanges that account for the average summertime salinity distribution in Great Pond (Barlow, 1952). These tidal exchanges must affect the horizontal distribution of plankton as well.

Ketchum, Ayers and Vaccaro (1952) have pointed out that if organisms such as coliform bacteria were introduced at a constant rate with the river water, their eventual distribution within an estuary would be similar to that of the fresh water, providing there were no net reproduction or mortality of the organisms. It may also be pointed out that these tidal exchanges must tend to disperse organisms landward. Hence, if plankton were present in constant numbers in Vineyard Sound its eventual distribution in Great Pond would be similar to that of the salt water, providing there were no net reproduction or mortality of the organism as it was dispersed through the pond.

Estuaries are not uniform environments. The salinity of Great Pond, ranging as it does from about 32 ‰ in Vineyard Sound to zero at the river mouth, provides in itself a significant environmental gradient. There must be similar gradients of other conservative environmental factors present in different quantities in the salt and fresh water.

It seems unlikely that there can be many organisms whose reproduction, growth and survival are not affected by such large environmental differences. We have found no zooplankton in Great Pond whose distribution landward or seaward from its center of abundance is consistently similar to that of the salt or fresh water. The distribution of the zooplankton is, therefore, determined not only by the exchanges of salt and fresh water but also by its own reproduction, mortality, and its specific behavior.

Ketchum, Ayers and Vaccaro (1952) have considered the effects of tidal exchanges and mortality on the distribution of coliform bacteria in the Raritan River. They have shown that after m tidal cycles the concentration C_n of coliform bacteria in a segment n of the river equal in length to one tidal excursion is

$$C_n = (C_0)_n \cdot r_n \cdot \frac{1 - (1 - r_n)^m e^{mk}}{1 - (1 - r_n)e^k}, \tag{1}$$

in which $(C_0)_n$ is the concentration of bacteria entering the segment n from the next landward segment and r_n is the exchange ratio of the segment n. The term e^k is the coefficient of net population change due to reproduction and mortality.

Since predation and the bactericidal action of sea water were always greater in their effects than reproduction, the sign of the coefficient k of the coliform bacteria was always negative. In some parts of the estuary reproduction will, however, tend to increase the size of the populations of many estuarine organisms. The sign of the coefficient k may thus often be positive.

Equation (1) can be simplified (see Ketchum, Ayers and Vaccaro, 1952) and solved for e^k to give

$$e^k = \frac{C_n - (C_0)_n(r_n)}{C_n(1 - r_n)}. \tag{2}$$

The simplification is valid only when $(1 - r_n)e^k$ is less than unity.

The term e^k in equation (2) defines the rate at which the population in a given segment must be increased to maintain it there at the concentration C_n in face of additions or withdrawals by tidal exchanges. This rate will be called the "replacement rate." If the sign of k in a specific segment of the estuary is positive, it implies that the population within the confines of that segment must be increasing by reproduction or by additions from deeper water below the limits of the segment to maintain the observed concentration in spite of the losses due to tidal exchanges. If the sign of k is negative, it implies that the population within the segment must be decreasing by mortality or by losses to the deeper water to maintain the observed concentration in spite of the gains due to tidal exchanges.

Estimates of the rates of the processes that maintain the observed distribution of Acartia

The replacement rates necessary to maintain a population at an observed steady state concentration C_n in any segment of Great Pond may be readily computed if the distribution of the population is well enough known that the contribution $(C_0)_n$ from the next landward segment can be estimated.

Both *Acartia* nauplii and *Acartia tonsa* copepodids were abundant and widespread throughout June and July, 1950. The *Acartia* copepodids were found in about the same abundance at the several stations in the pond on two successive surveys, June 30 and July 10, and the nauplii on three successive surveys, June 5, 15 and 30.

The nauplii were most abundant at the northern end of the main pond and in the arm, at stations 4, 5 and 5a, during June. An analysis of variance (Snedecor, 1946) of the June counts of nauplii at these three stations showed that none of the differences between surface and bottom concentrations, or between surveys, were significant. There were similar differences in abundance of *Acartia* copepodids in the June 30 and July 10 surveys. The distribution of these two forms appears to have been approximately in a steady state for the periods covered by these successive surveys.

The concentrations of *Acartia* nauplii and copepodids in the tidal excursion segments of the pond were estimated from the data of these surveys and are shown in Figures 4 and 5. At stations where samples were taken at both surface and bottom the average of surface and bottom concentrations has been considered to most nearly represent the average concentration in the mixed layer. The replacement rates necessary to maintain in each segment these observed steady state concentrations of *Acartia* nauplii and copepodids have been computed, using the relationship presented in equation (2). The replacement coefficients are shown in Figures 4 and 5. Each of these coefficients has been plotted against the mean salinity of its respective segment.

At salinities less than 15 ‰, representing values from the arm landward of station 5, the replacement coefficients were usually positive and relatively large. These large coefficients indicate that the concentrations of both nauplii and copepodids in the mixed layer of the arm were being maintained in face of seaward transport by very rapid replacement.

At the intermediate salinities of from 15 ‰ to 25 ‰, representing values from the narrows and the northern end of the main pond, the replacement coefficients were also usually positive but much smaller than in the arm. These coefficients indicate that, even though the populations were large in this part of the pond, they were being maintained by replacement at a moderate rate.

At salinities above 25 ‰, representing values from the southern half of the main pond and the shallows, the replacement coefficients were nearly all negative. These coefficients indicate that the *Acartia* must have been removed from this part faster than could be accounted for by exchanges with the Sound.

Production rate in isolated water samples

The replacement rates, c^k, calculated from equation (2) represent the rates of change in the population by reproduction and mortality which, under steady state conditions, balance the gains or losses by tidal exchanges. These rates would

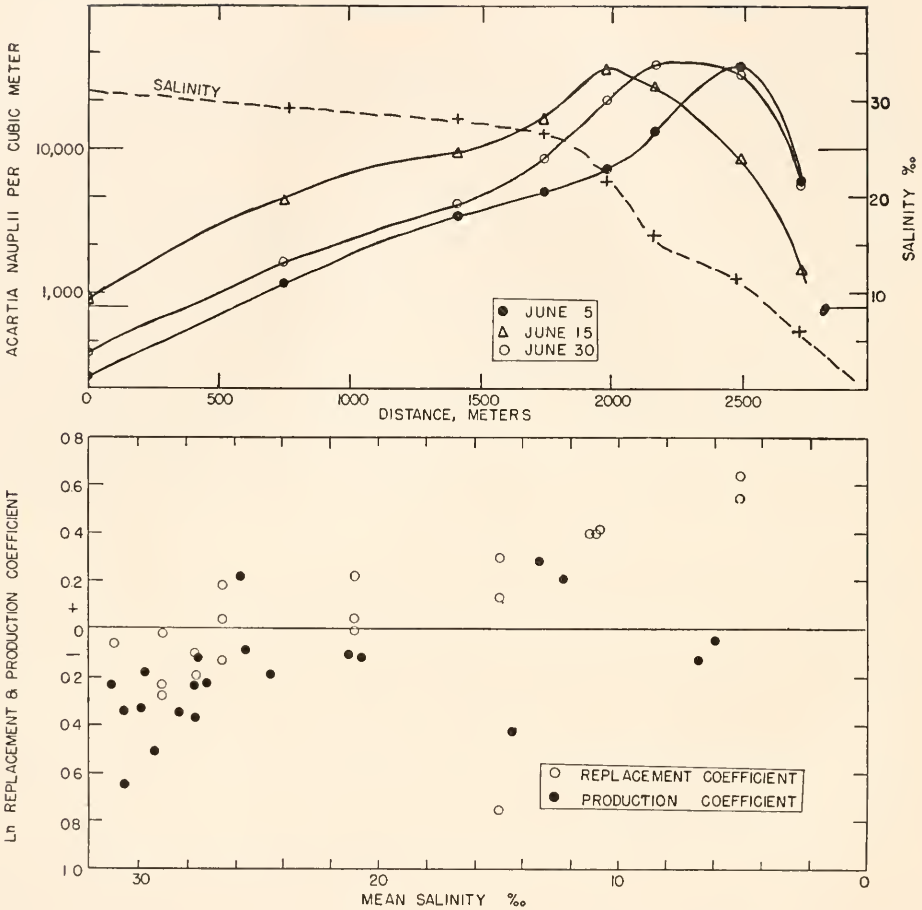

FIGURE 4. Distribution of *Acartia* nauplii (above) and replacement and production coefficients for *Acartia* nauplii (below). Replacement coefficients calculated from distributions of June 5, 15 and 30, 1950. Natural logarithms of replacement rates, e^k, plotted against mean salinity in tidal excursion segments. Production coefficients calculated from bottle experiments of June and July 1951. Natural logarithms of production rates, e^k, plotted against salinity in the bottles.

also reflect the effects of vertical exchanges between the mixed layer and the deeper layer, which may or may not be significant. The replacement rate thus gives an estimate of the rate at which the population would increase or decrease if purely horizontal tidal exchanges were eliminated.

An independent estimate of the value of e^k may be obtained by actually isolating a sample of water with its natural population from tidal exchanges and noting the rate at which its population actually changes when maintained under otherwise natural conditions. These two estimates should agree unless vertical exchanges are significant.

The change in numbers in an isolated parcel of water may be represented by

$$(C_0)e^{kt} = C_t, \tag{3}$$

in which C_0 is the initial concentration of the organism and C_t the concentration after t tidal cycles. The value of e^k determined from equation (3) will be called the "production rate," in distinction from the value obtained from equation (2) which is referred to as the replacement rate.

When applied to the numbers of some particular stage in the life history of an organism, as in the present case, these production rates are not, of course, true

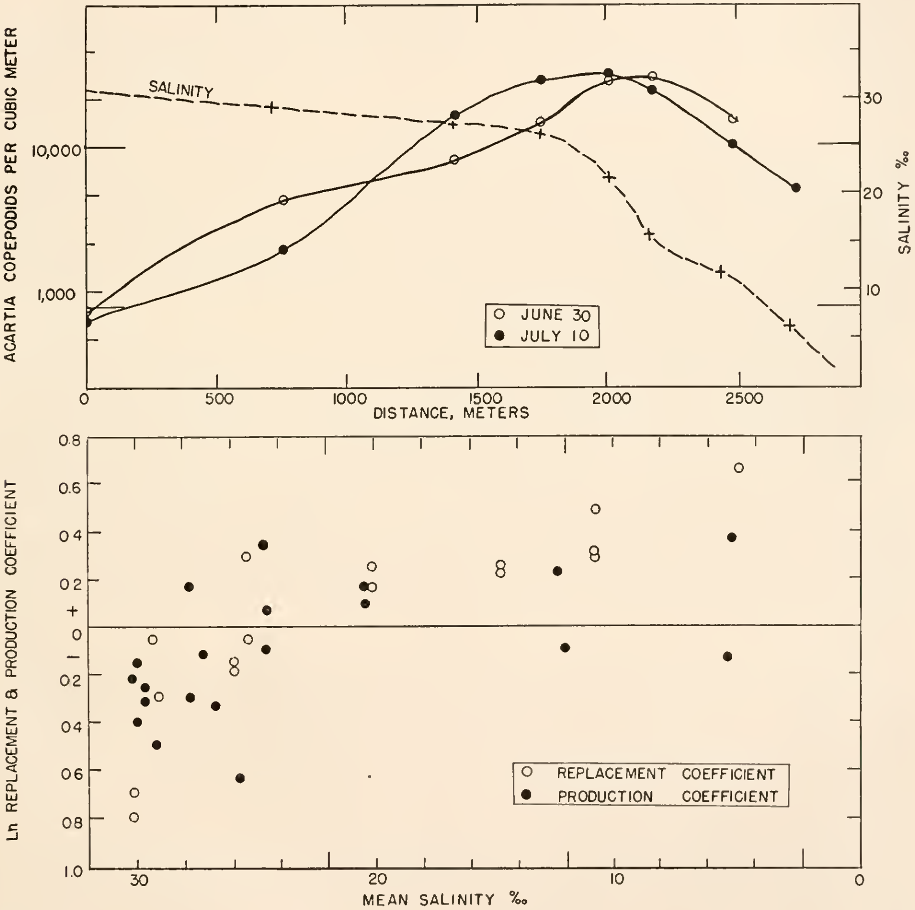

FIGURE 5. Distribution of *Acartia tonsa* copepodids (above) and replacement and production coefficients for *Acartia tonsa* copepodids (below). Replacement coefficients calculated from distributions of June 30 and July 10, 1950. Natural logarithms of replacement rates, e^k, plotted against mean salinity in tidal excursion segments. Production coefficients calculated from bottle experiments of June and July, 1951. Natural logarithms of production rates, e^k, plotted against salinity in the bottles.

measures of the rate of change of the entire population; they refer rather to the changes resulting from hatching, moulting, and the mortality of the particular stage in question.

In the bottle experiments portions of the *Acartia* population were effectively isolated from all exchanges with the surrounding water, but were maintained under natural conditions of temperature and illumination. Production coefficients have been computed from the change in numbers of *Acartia* nauplii and copepodids during these experiments using equation (3). Since it is reasonable to assume that the environmental conditions in the pond were essentially the same in 1950 and 1951, these production coefficients have also been shown in Figures 4 and 5 for comparison with the replacement coefficients. The production coefficients have been plotted against the salinity of the water enclosed in each bottle.

Comparison of the production rates with the replacement rates shows that in the main pond these two estimates of the rates of the biological processes are in general agreement. The greatest production rates were at the intermediate salinities of 10 to 15 $^0/_{00}$, typical of the narrows and northern end of the main pond. There the populations often reproduced fast enough to double themselves in less than two days. The agreement between the replacement and production rates indicates that there the *Acartia* population was replaced in the mixed layer by reproduction and by horizontal tidal exchanges with the population in the more landward part of the pond. The deeper layer could not have been a very potent source of recruits to the mixed layer since the *Acartia* population in the deeper layer in this part of the pond was little larger than that in the mixed layer.

At the higher salinities typical of the more seaward part of the pond the production coefficients were usually negative, showing that the *Acartia* brought in by exchanges with the landward parts of the pond were being reduced. Since the replacement rates were also negative these populations must have been reduced by some other means than by exchanges with the Sound. The hydrographic data show that most of this part of the pond was so thoroughly mixed that there could seldom be a distinct deeper layer which could receive the *Acartia* removed from the mixed layer. These populations must, therefore, have been reduced by an excess of mortality within this part of the pond over recruitment from the landward parts of the pond.

The cause of this mortality in the southern end of the pond is not known. Similar mortality rates have, however, been shown for lake zooplankton caught in outflowing rivers. Galtsoff (1924) has suggested that the mortality of this lake zooplankton may often be caused by the strong turbulence in the river. The strong turbulence in the shallows and entrance may have caused an excessive mortality of zooplankton in that part of the pond.

The replacement rates at the low salinities typical of the mixed layer of the arm were much higher than the production rates. These replacement rates indicate that the populations in this part of the pond must have been replaced very rapidly if they were to be maintained there. If they were to be replaced by reproduction alone, they would have had to double or triple themselves each day. As the rates of increase in the bottle experiments suggest, these are improbably high rates for an organism such as *Acartia*. A large part of the copepod population in the mixed layer may have been recruited by vertical mixing or migration from the deeper layer. This is possible because the deeper layer usually had a much richer population than the surface layer. The population in the deeper layer in turn must have been replaced rapidly if it were to provide a continuous supply to the mixed layer. This replacement may have been provided by landward transport in a deeper counter current from the regions of maximum abundance near Station 5.

The comparison of the replacement and production rates made above has shown that a high rate of reproduction served to maintain the populations in the narrows and northern end of the main pond. At higher salinities, in the more seaward parts of the pond, there was a decrease in the rate of the biological processes. The small populations in these parts of the pond were a result of mortality and exchanges with the Sound. At the lowest salinities, in the surface layer of the arm, the biological processes are insufficient to maintain the populations found there. The discrepancy between the rate at which these organisms reproduce and the rate at which they must be replaced in these parts of the pond appears to be made up by recruitment from the deeper layer.

Discussion

The distribution of plankton does not in itself give a reliable definition of the limits of the region in which the organism is able to maintain itself endemically by its own reproduction and growth. The analysis of the processes that maintain the distribution of *Acartia* has shown that nowhere in the surface mixed layer of Great Pond did this copepod maintain itself wholly by local reproduction. The population in the arm could not have maintained itself without continuous additions from the deeper layer. Even the population in the mixed layer of the northern end of the main pond was dependent to some extent on additions from the more landward parts of the pond. Mortality must have far exceeded reproduction to account for the observed concentrations of *Acartia* in the seaward part of the pond.

If any holoplankton organism is to attain an endemic relation to a specific region, it is necessary that the biological processes that maintain it balance the physical processes that disperse it. This necessity is lessened but not evaded by organisms seeking regions in which dispersion is less rapid such as the deeper layer in Great Pond. In the final analysis this necessity can be met only by a sufficient rate of population increase. The fitness of a plankton organism to establish an endemic relation to a specific region depends on its rate of reproduction and growth and on its survival in the environment of that region. Smaller organisms often have the higher reproductive rates and shorter periods of immaturity that contribute to high rates of population increase. It is perhaps significant that all the endemic plankton organisms of an estuary such as Great Pond are small.

The physical processes of advection and diffusion set the minimum rate at which a plankton organism must be able to increase to maintain itself endemically. In Great Pond this minimum rate was achieved by several zooplankton in the summer during the time when temperature is high and the phytoplankton abundant. In the late summer and early fall these zooplankton appear to be unable to reproduce fast enough to maintain their numbers, perhaps because of the depredations of ctenophores.

The higher salinities in the pond in the winter suggest that there may have been some change in the physical processes that dispersed the plankton. Whether the rate of these physical processes increased or not, the biological processes of reproduction and growth must have been decreased by the lower temperatures and sparser phytoplankton of the winter to the point where they may have been insufficient for any zooplankton to maintain themselves in the estuary by these processes. The zooplankton would, however, be able to tide themselves over such periods by the formation of resting stages on the bottom. It was not until the temperature and the phytoplankton began to increase in the spring that there was any clear evidence that any of the zooplankton in the pond was able to increase sufficiently to maintain itself endemically.

SUMMARY

1. The zooplankton of a small estuary, Great Pond, has been described. It has been shown that in the summer there were endemic populations of several zooplankton of which the copepod *Acartia* was the most numerous and widespread.

2. The rates at which *Acartia* must have been replaced to maintain its numbers in the surface mixed layer of Great Pond in face of dispersion by the tidal exchanges have been calculated. These rates are compared with the rates at which *Acartia* increased or decreased when isolated in bottles immersed in the pond.

3. This comparison shows that in the most landward parts of the pond the population in the mixed layer could not reproduce fast enough to maintain itself and was dependent on recruitment from the deeper layer. In the central part of the pond reproduction alone was sufficient to offset the seaward transport by the tidal exchanges. In the most seaward parts of the pond, mortality was so great that the small population was maintained only by transport from the landward parts of the pond.

4. Thus the comparison reveals the contribution of reproduction, growth and mortality and of tidal transport to the maintenance of the observed distribution of *Acartia*. Seasonal changes in these physical and biological processes account for the seasonal changes in the distribution of zooplankton in Great Pond.

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THE ANNUAL REPRODUCTIVE AND NUTRITIONAL CYCLES IN TWO WESTERN SEA URCHINS[1]

JOHN BENNETT AND ARTHUR C. GIESE

Hopkins Marine Station, Pacific Grove, California

A previous study of the reproductive state of the purple sea urchin, *Strongylocentrotus purpuratus,* suggested the possibility of more than one cycle of gonadal growth during the year 1952–53 (Lasker and Giese, 1954). The reproductive state was assayed by determining the ratio of the volume in ml. of gonadal tissue to the wet weight in grams of the sea urchin, this ratio being called the gonad index (GI). In order to obtain more decisive data on gonadal growth, in the present study the gonad index was determined twice monthly for a year (1953–54) except for a few times when poor tides made collection hazardous. Furthermore, samplings were made at two localities, Pescadero Point on the Monterey Peninsula, and Yankee Point, a more exposed location on the Pacific Ocean about 10 miles farther south, since in this way the effect of different local conditions might be checked. Each sample consisted of 20 animals; thus gonad indices were obtained for 20 specimens per fortnight from each of two collecting grounds.

A monthly study of the gonad index of the large sea urchin, *Strongylocentrotus franciscanus,* was also made in 1953–54, in order to see to what extent its reproductive cycle resembled that of the smaller urchin. Collection was limited in this case not by tides but by conditions on the water; when the surface was rough, collection from a boat became too difficult. Therefore, though no collections were missed, the time intervals between collections were not perfectly regular. Ten specimens of *S. franciscanus* were analyzed each month, from catches obtained with a large net dragged by rope in about 15 feet of water near the Hopkins Marine Station.

The previous study involved the determination of nutritive state as given by the body fluid non-protein nitrogen (NPN) and reducing sugar (RS) in every specimen of *S. purpuratus* whose gonad index was determined. The same thing was done in this study for every specimen of *S. franciscanus.* Time limitations prevented the continuation of NPN and RS measurements in *S. purpuratus,* however, only occasional samples being taken.

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Experimental Data

1. *The reproductive cycle of S. purpuratus*

The data for the gonad index of the purple sea urchin for the year 1953–54 are given in Figures 1 and 2, along with similar data for 1952–53. It is evident that only a single breeding cycle occurred at Pescadero Point during 1953–54, whereas the data from the same location in 1952–53 suggest the possibility of a second cycle. At Yankee Point there was a dip in February, 1954 which appears to correspond to that at Pescadero Point in February, 1953; but this may be spurious, since it is due to the presence of 8 spawned-out animals among the 20 of the sample, the

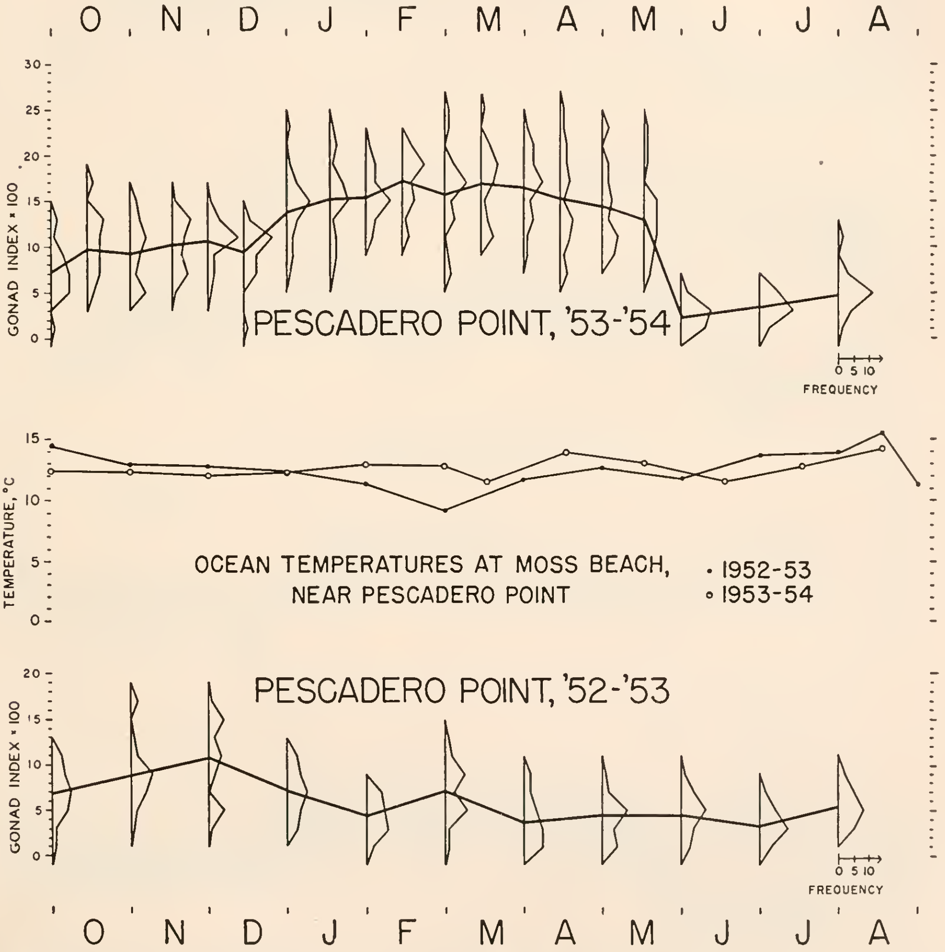


Figure 1. Reproductive cycle of *Strongylocentrotus purpuratus* in succeeding years, and corresponding ocean temperatures. The great difference in ripening is correlated with an opposite temperature gradient in the winter months. Each sample distribution is plotted in full, and a solid line joins the sample means. The frequency scale is the same for each distribution.

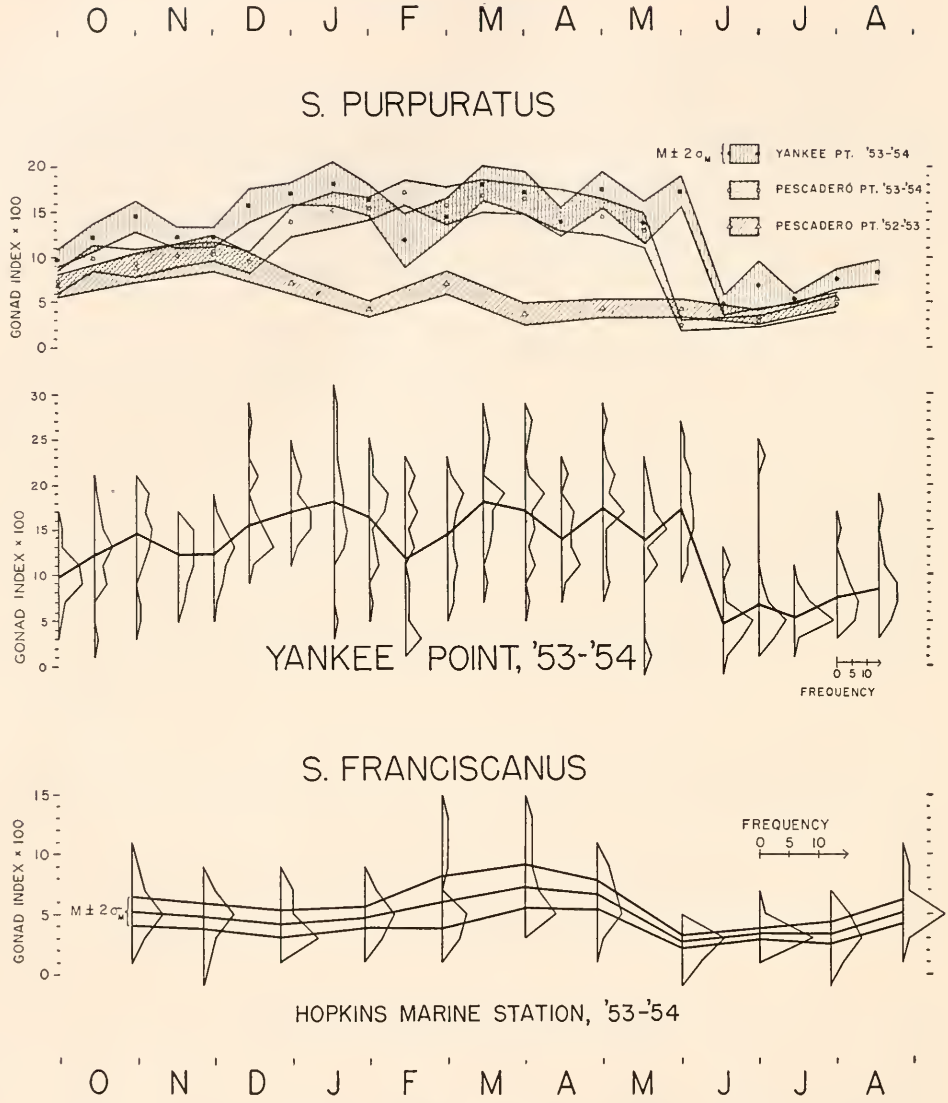
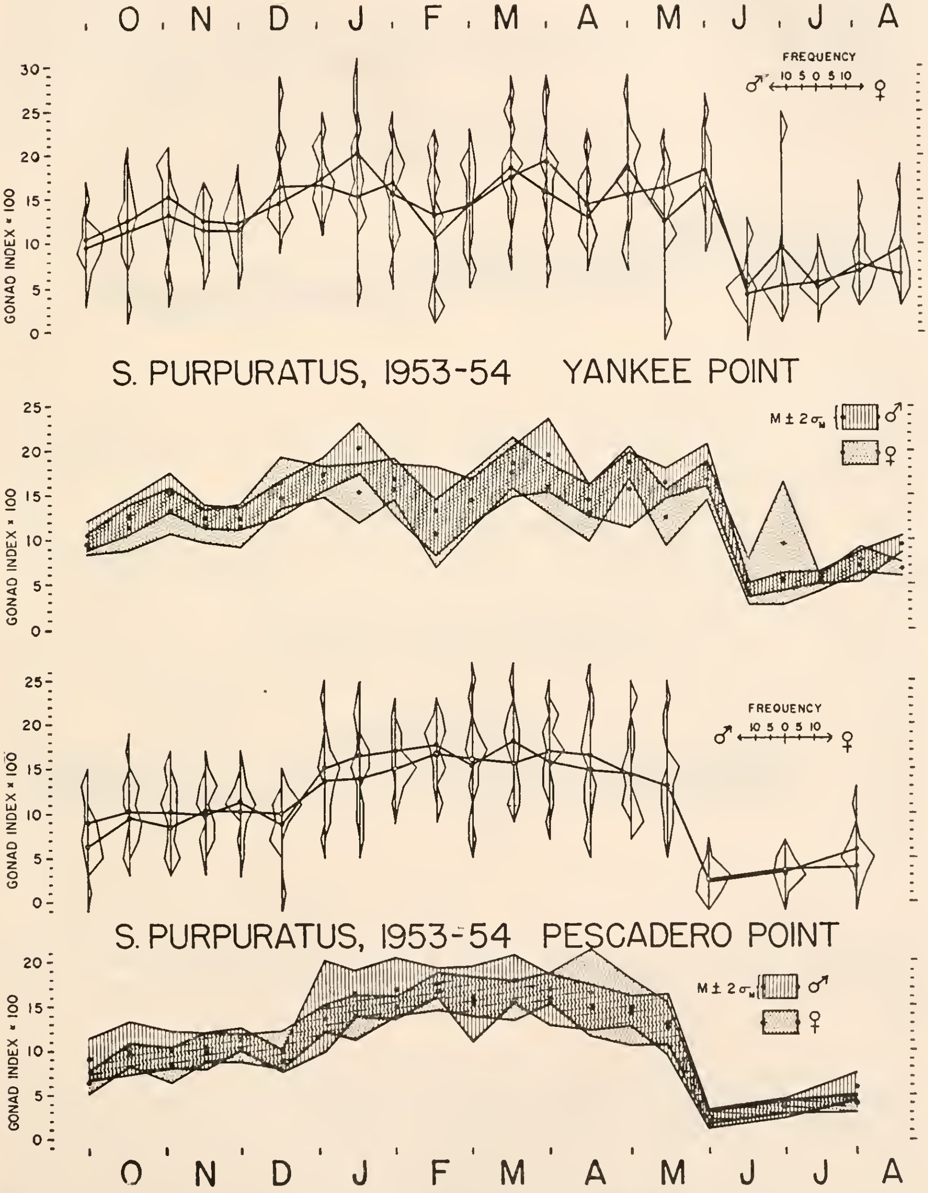


FIGURE 2. Reproductive cycles of *S. purpuratus* and of *S. franciscanus.* The bands in the top chart represent approximately 95 per cent confidence limits about the sample means; thus high significance of the differences between 1952–53 and 1953–54 means is clearly indicated. A similar band is drawn in the bottom chart to show the degree of significance of the seasonal variations in *S. franciscanus.*

other 12 being fully as gravid as the animals at the peak periods. All things considered, it therefore seems more reasonable to assume a single annual breeding cycle for the species with random variations, than a consistent double or multiple cycle.

The most striking difference between the 1952–53 and the 1953–54 cycles is the much higher gonad indices for the latter year. The gonads reached almost double their 1952–53 size. It would be very interesting to know to what factor this greater growth might be attributed, for example whether it was a better food supply or a more favorable temperature. Examination of the temperature data shown in Figure 1 indicates that the ocean temperatures, in general, were higher



S. PURPURATUS, 1953-54 YANKEE POINT

S. PURPURATUS, 1953-54 PESCADERO POINT

Figure 3. Male and female reproductive cycles of *S. purpuratus* (1953–1954). The overlap of the 95 per cent confidence bands throughout the year indicates a close correlation of male and female cycles.

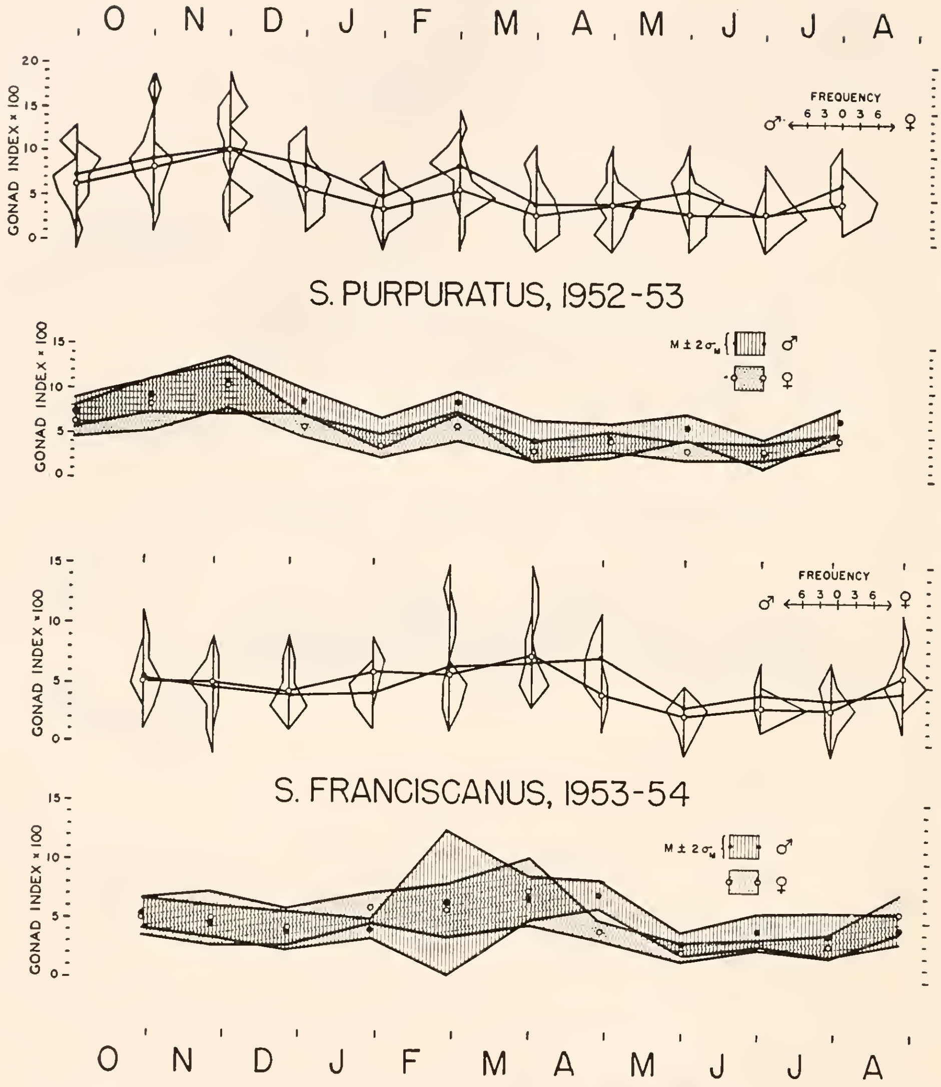


FIGURE 4. Male and female reproductive cycles of *S. purpuratus* (1952–1953), and of *S. franciscanus* (1953–1954). Again no significant difference between male and female cycles is shown.

in 1953–54 than in 1952–53. This could have had an indirect effect on the urchins by making possible a larger growth of algae. Unfortunately, no data are at present available on the algal crop for the year. Other physiological variables have been considered as possible causes of the observed difference, *e.g.* rainfall (therefore indirectly, salinity), and amount of sunlight; but there seems to be less difference between the two years on the basis of these factors than on the basis of water temperature, perhaps correlated with algal growth.

The data for the reproductive cycle in male and female urchins are separately analyzed in Figures 3 and 4. While male gametes are available for a longer period of time than female, it is evident that only a single, closely correlated cycle of gonad index occurs in both sexes.

The fertilizability of eggs was determined at each sampling, except during the peak months of 1953–54 when nearly every animal exuded great masses of gametes, which condition was previously and subsequently found to be an almost certain indication of ripeness. The criterion of ripeness in females was development of fertilization membranes in over 50 per cent of eggs in the microscope field after introduction of sperm. In males, the criterion was simply presence of sperm, there being found no instance of motile sperm which would not fertilize ripe eggs.

The difference in gonad index between the 1952–53 and 1953–54 seasons was strongly correlated with a difference in ripeness of females; in no collection during 1952–53 were a majority of the females ripe, whereas practically all females were ripe from December through May of 1953–54. The males did not provide as good a contrast, though there were many more unripe males found during the earlier year.

It is a point of interest that starvation does not appear to decrease fertility in these urchins. In fact, one of the most fertile females observed was a "spawned-out" animal which had been starved for three weeks and which appeared near death, most of its spines having fallen off. It exuded many eggs, virtually all of which developed excellent membranes within a few seconds after sperm were provided.

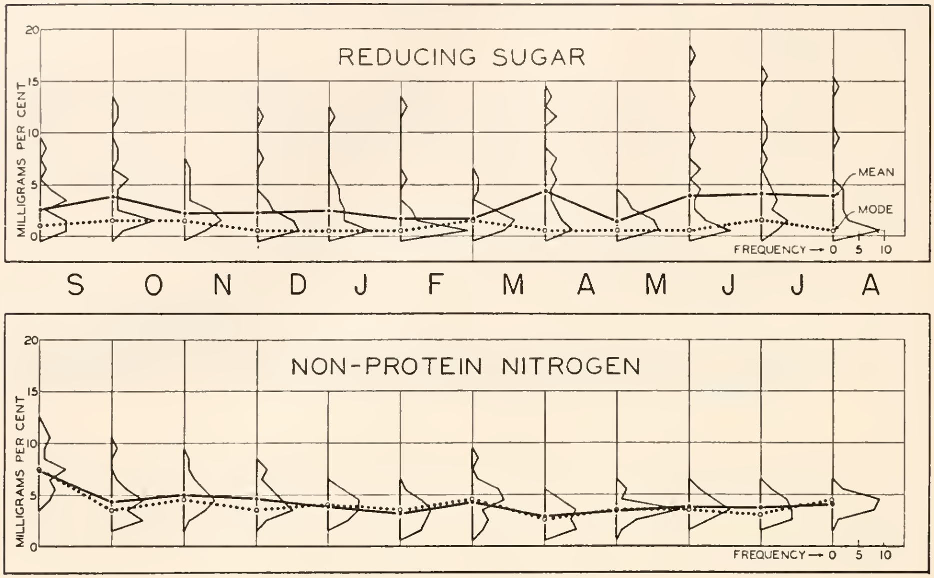
2. *The reproductive cycle of S. franciscanus*

A study of the reproductive cycle was also made for the large sea urchin, *S. franciscanus,* and the data for the gonad index are shown in Figure 2. A single cycle for the year is manifested, but the index reached its maximum somewhat later than in the purple sea urchin. The index was high for a much briefer period of time, suggesting a shorter breeding season. The gonad index of the larger species also never reached the large values found in the smaller species during the year. Perhaps because of the size of the animals, an equivalent enlargement of gonad would be beyond the aeration capacity of the body fluid, although no decrease of gonad index with increasing size was observed in *S. purpuratus*. Fertilizability of eggs of *S. franciscanus* was determined in the same way as for the smaller species, except that the eggs were followed to cleavage because the fertilization membranes were indistinct in some cases. Ripeness was found to be correlated with gonad index, better in the case of females than males, just as in *S. purpuratus*. But no such tremendous fecundity was exhibited by *S. franciscanus* as by the smaller species during 1953–54. A close correlation of male and female cycles of gonad index was found in *S. franciscanus,* as in *S. purpuratus* (Fig. 4).

3. *The NPN and RS cycles in S. purpuratus*

The techniques employed in measuring NPN and RS in *S. purpuratus* have been described in the previous study (Lasker and Giese, 1954). The results were reported by graphing the means of the monthly samples, but the descriptive statistical work undertaken in the present study has revealed a feature of the earlier results which calls for their analysis and further discussion. The data are represented in Figure 5, with the distribution of each of the monthly samples super-

S. PURPURATUS, 1952-53



S. FRANCISCANUS, 1953-54

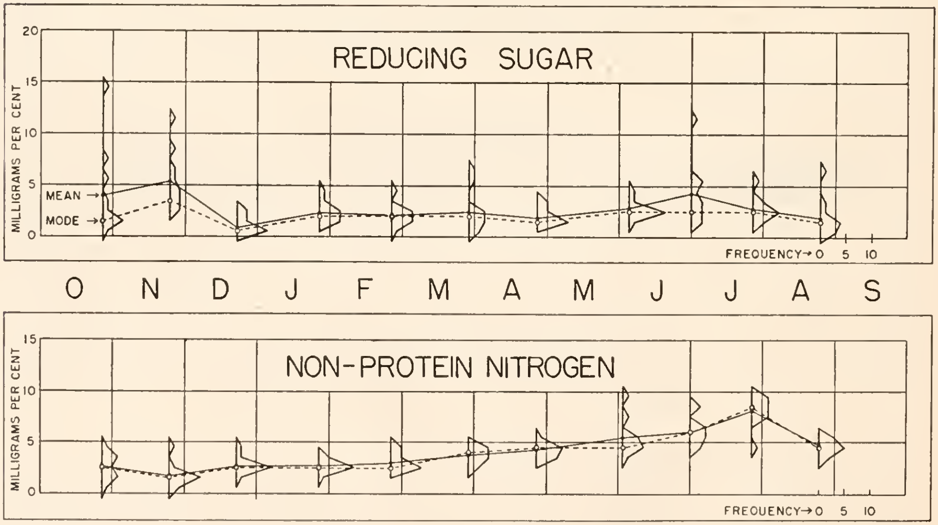


FIGURE 5. Reducing sugar and non-protein nitrogen cycles in *S. purpuratus* and *S. franciscanus*. RS distributions are positively skewed and NPN distributions are non-skewed. This is well indicated by the fact that mean is above mode in every RS distribution, but as often below as above in the NPN distributions.

posed on the graphs of means, and with the graphs of modes also included. It is clear that no highly significant seasonal variation is indicated in either NPN or RS in this species. The outstanding feature is the difference in skewness of the two kinds of distributions, the NPN being fairly normal, while the RS are highly skew.

This difference in shape of NPN and RS distributions is a fact which might have remained undiscovered had the routine statistical work of plotting the distributions not been done. It thus illustrates in a small way the importance of adequate descriptive statistical treatment of experimental data.

It is quite apparent that in the RS distributions the mean does not represent the point where the bulk of each distribution is concentrated, as it is usually assumed to do, and that the bulk of the distribution is more stable than the mean, so that there is less seasonal variability in RS than is indicated by a graph of the means. This is an instance in which the mode, which by definition follows the bulk of the distribution, provides a less misleading representation of the data than does the mean.

In the NPN distributions, by contrast, mean and mode closely coincide in almost every case, and for statistical reasons the mean is to be preferred for the representation of the data.

4. *The NPN and RS cycles in S. franciscanus*

NPN and RS in *S. franciscanus* were determined during 1953–54 by the same basic techniques as those employed with *S. purpuratus,* and the results are represented in the same way as for the latter species in Figure 5. The differences in skewness of distribution of NPN and RS appear in this species also. The difference is not so striking, but this may be an entirely statistical effect, owing to the smaller sample size.

Whereas no seasonal variations were clearly demonstrated in *S. purpuratus,* there was a decidedly significant increase in NPN throughout the spring in *S. franciscanus.* No correlation was observed between the growth of gonads, as measured by the gonad index, and the amount of NPN in the body fluid. The cause of the seasonal variation of NPN in *S. franciscanus* thus remains undetermined, though it may be associated with the great outgrowth of algae which occurs during the summer.

5. *Total nitrogen in the filtered body fluid of the two species*

Total nitrogen was measured, as well as NPN, in the filtered body fluids of a few specimens of each species, the differences being attributable to proteins. Results are shown in Table I. Technical limitations were such that accurate figures for the protein N could not be obtained; it can be said only that the protein N is a small percentage of the total, almost certainly less than 20 per cent. Thus the protein concentration, excluding clotting proteins which were always removed in filtration, is of the order of 0.5 mg. per cent.

Discussion

An annual breeding season, as judged by spawning, is believed to be common in many west coast invertebrates (MacGinitie and MacGinitie, 1949). Field observations of spawning have also established that the breeding span for a given

TABLE I

Mg % total nitrogen and protein nitrogen in the body fluid of Strongylocentrotus purpuratus and S. franciscanus

A. *S. purpuratus.* $M \pm 2\sigma_M$ from duplicate determinations

Total N	5.90 ± .14
NPN	5.43 ± .14
PN	0.47 ± .20

B. *S. franciscanus.* $M \pm 2\sigma_M$ from duplicate determinations

Total N	2.46 ± .17	4.79 ± .17	3.08 ± .17
NPN	2.04 ± .28	4.30 ± .14	2.46 ± .06
PN	0.42 ± .32	0.49 ± .22	0.62 ± .18

C. *S. franciscanus.* $M \pm 2\sigma_M$ from triplicate determinations

Total N	6.71 ± .31	5.76 ± .33	3.86 ± .14	5.60 ± .21
NPN	6.31 ± .09	4.90 ± .14	3.50 ± .07	5.21 ± .06
PN	0.40 ± .32	0.86 ± .36	0.36 ± .16	0.39 ± .22

species is different at different geographical locations along the coast, and at a given location, for different habitats, *e.g.,* in deeper water as compared to shallow. However, the span of the season as defined by gonad size has not been characterized in many species.[2]

In the present account which reports a series of measurements on gonads from two species of sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus,* there is indicated a single reproductive season during which the gonads gradually increase in size until, in each individual of the former species taken from a given habitat, they are so swollen with gametes as virtually to fill the body cavity. They remain in this condition until induced by some undetermined cause to spawn, and then the gonads become spent throughout the entire local population in a fairly short time. While it is likely that gonads may become replenished in the course of a season, no conclusive evidence for this was obtained, since the same animals could not be sampled successively. Spawning might give some indication of this possibility, but no satisfactory method for its induction in the laboratory was found, although chemical and electrical methods were tried (Harvey, 1939, 1954).

The factors involved in the growth of the gonads may be numerous. That temperature influences productivity is suggested by the doubled gonad volume reached during 1953–54 as compared to that of 1952–53, the former being a warmer year with no very cold spells in the water. A correlation with temperatures was reported by Stott (1931) for the sea urchin *Echinus esculentus,* spawning occurring when the sea temperature is rising in late April, May, and June. He observes the same for *Mytilus* and *Ostrea* in the same region (Port Erin). Such an effect might act indirectly by affecting the growth of the algae. More data on growth of gonads in many species of invertebrates, in addition to information on their spawning, are desirable before correlation with climatic factors can be made significant.

It is a natural presumption that cycles of nutritional components of the body fluid would be correlated with the reproductive cycle in many marine invertebrates.

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[2] This has been done with vertebrates; see Bullough (1951).

This was not found to be the case with the species studied here, however. No seasonal variations in reducing sugars were found in either species; non-protein nitrogen varied seasonally in *S. franciscanus,* but no relation between the breeding and NPN cycles was established.[3]

The skewness of RS and the normality of NPN distributions in both species are facts which call for an explanation. Even neglecting the shapes, the differences in variability of these distributions suggest that NPN is loosely regulated, perhaps in association with the gonads, while RS may be entirely unregulated in the organism. As for the shapes themselves, a possible interpretation of the skewness of the RS distributions is that only a small proportion of urchins is feeding at any given time, this proportion having a relatively high RS concentration during the short period of the transfer of readily available sugars across the gut wall, while the non-feeding majority have very little RS in the body fluid, because of its rapid absorption by tissues.

Various aspects of this interpretation are subject to experimental testing. In the first place, starvation experiments should reveal the differences in degree of regulation of RS and NPN. Results of such experiments, reported in the previous paper (Lasker and Giese, 1954), demonstrate the greater regulation of NPN, and the virtually complete absence of regulation of RS.

In the second place, the role of the gonads in NPN regulation may be investigated by means of starvation experiments on gravid urchins and spawned-out urchins. Regulation should be superior in the former if the gonads play a major role. An attempt to carry out this experiment was only partially successful, because the unripe animals were not completely spawned out, as revealed at the end of the starvation period (Table II). The increase of mean NPN of specimens fed for one and two days after the starvation period shows that the regulation of NPN is rather loose.

In the third place, the proportion of specimens taken in the act of feeding on sugar-rich algae could be counted, and their RS concentrations determined separately, as a partial check on the interpretation of RS skewness; such animals should constitute a small proportion, and should have a relatively high RS concentration if the interpretation is correct. This work remains to be done but is retarded because of lack of information on the algae.

Finally, the RS skewness depends also on the rapid uptake of RS from the body fluid by the tissues. A determination of the time of withdrawal of injected glucose, reported in the previous study, indicates that over 90 per cent of the glucose is withdrawn within 4 hours; this result supports the suggested interpretation.

Other interpretations are possible, which may be in accord with the known facts in whole or in part. One is that the RS skewness may be due to the presence of a metabolic disorder, analogous to diabetes, in a small proportion of the animals. A simple experiment would decide between this and the former interpretation: If a sample of animals were fed boiled potato, only a small proportion should have a marked increase of RS if the metabolic disorder interpretation is correct, while all should have it if the other is correct. Results of the previous study (see Table I, Lasker and Giese, 1954) show marked increase in RS in three out of four animals thus fed, supporting the original interpretation, though not conclusively.

Further work remains to be done before it can be said that the shapes of the

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[3] Similar results are appearing in the present season 1954–55.

Table II

NPN and RS in the body fluid of S. purpuratus after starvation and refeeding boiled egg white

GI*	Wet weight of urchin in grams	Weight ingested in grams	Mg % RS	Mg % NPN ($M \pm 2\sigma_M$)	Gonad N % ($M \pm 2\sigma_M$)
A. Five urchins of low GI, starved for three weeks					
3.3	70.5	0	0.6	5.3 ± 0.4	1.93 ± .05
4.8	77.9	0	0.7	6.2 ± 0.4	1.92
3.1	77.3	0	0.4	5.4 ± 0.4	1.95 ± .42
4.3	94.9	0	0.5	6.7 ± 0.4	2.25 ± .02
2.9	91.4	0	0.8	6.1 ± 0.4	2.07 ± .18
3.7 (av.)			0.6 (av.)	5.9 (av.)	
B. Four urchins of high GI, starved for three weeks					
16.7	67.1	0	1.5	2.7 ± 0.2	
15.0	92.2	0	1.9	4.2 ± 2.8	
15.8	51.1	0	3.4	5.5	
10.5	93.1	0	2.0	3.7 ± 1.0	
14.5 (av.)			2.2 (av.)	4.0 (av.)	
C. Four urchins of high GI, starved three weeks, then fed one day					
13.9	83.0	2.67	3.1	6.1 ± 1.2	
10.2	56.0	1.21	3.3	7.4 ± 2.0	
12.6	70.6	2.75	3.2	6.8 ± 0.6	
13.1	69.2	3.22	—	10.6	
12.5 (av.)			3.2 (av.)	7.7 (av.)	
D. Three urchins, starved three weeks, then fed two days					
5.4	51.8	6.50	3.5	9.2	
2.4	77.8	5.06	3.4	8.2 ± 0.2	
15.6	85.1	5.40	3.2	7.5 ± 0.2	
7.8 (av.)			3.4 (av.)	8.3 (av.)	

* GI (gonad index) as used for these data is the ratio of gonad weight in grams (instead of gonad volume in cc.) to urchin wet weight in grams.

RS and NPN distributions in these urchins are fully understood. It can be said now, however, that the shapes of the distribution curves are facts of some interest, and suggestive of interpretations which can be subjected to further investigation. Moreover, the systematic investigation of the shapes of distributions of various components of body fluid in other invertebrates may afford a valuable supplement to starvation and feeding experiments in the investigation of regulation and its evolution in the blood.

Summary

1. The degree of gonadal development in the purple sea urchin, *Strongylocentrotus purpuratus,* was measured by taking the gonad index, or ratio of volume of gonad to wet weight of urchin, every two weeks for a year (1953–54) in samples of 20 urchins obtained from each of two localities, Pescadero Point on the Monterey Peninsula, and Yankee Point, 10 miles south.

2. For *S. purpuratus* from both places, the average gonad index rose during the fall to a peak twice as high as that reached the previous year, and this peak was maintained at a fairly constant level from January through May.

3. Climatic factors, rather than ecological, are therefore thought to have the greater effect upon gonad growth. A clear-cut correlation was not established for any factor, but ocean temperature is known to have been higher during the winter of 1953–54 than in the previous year.

4. Determination of the gonad index for monthly samples of 10 specimens of the large western sea urchin, *S. franciscanus,* taken from below the tide pools of the Hopkins Marine Station, indicated a single reproductive cycle, but a much shorter breeding season, the peak of which was reached later than that of *S. purpuratus.*

5. Though males were found to have fertile gametes for a greater part of the year than females, no significant difference was found in the reproductive cycles of male and female urchins in either species, as measured by gonad index.

6. The great growth of gonads was not correlated with an increase in NPN (non-protein nitrogen) or RS (reducing sugar) in the body fluid of either species. However, greater mobilization, *i.e.,* transport from gut to gonad, could not be determined by the methods used.

7. No seasonal variation of RS was found in either species. NPN was also invariant in *S. purpuratus,* but showed a marked increase in *S. franciscanus* during the summer.

8. In both species there was a much greater variability of RS than of NPN, suggesting no regulation of RS, but a degree of regulation of NPN. RS was skewly distributed, while NPN was normally distributed in nearly all samples, a fact which may be partially explained by the feeding habits of the animals.

9. Very small quantities of protein nitrogen of the order of 0.5 mg. per cent were found in the filtered body fluids of a few specimens of each species.

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EVIDENCE FOR AN EXOGENOUS CONTRIBUTION TO PERSISTENT DIURNAL AND LUNAR RHYTHMICITY UNDER SO-CALLED CONSTANT CONDITIONS[1]

F. A. BROWN, JR., H. M. WEBB, M. F. BENNETT AND M. I. SANDEEN

Department of Biology, Northwestern University, Department of Physiology, Goucher College, Department of Zoology, Duke University, and the Marine Biological Laboratory, Woods Hole, Massachusetts

Daily rhythmicity which persists in conditions of constant darkness and temperature has been reported for great numbers of animals and plants ranging from the simplest one-celled ones to the most complex multicellular ones. One of the most striking aspects of this rhythmicity is that it has been reported to continue with great precision, retaining its normal phase relationships with respect to the external day-night cycle over long periods of time. Since most of the observations have been made following the placing of animals in darkness at some arbitrarily selected temperature, virtually all of the studies have implied a temperature-independence of the mechanism responsible for maintaining the frequency of the daily cycles over at least a few degrees. A critical study of the temperature relationships of the daily rhythmicity in fiddler crabs (Brown and Webb, 1948) confirmed the temperature-independence of the frequency over the range, 6° to 26° C. At temperatures of 0° to 2° C. the mechanism responsible for regulating the frequency of the color change rhythm in the fiddler crabs was greatly depressed. More recently (Webb, Bennett, Graves and Stephens, 1953), it has been found that there is a daily rhythm in the degree of depression of the frequency of the mechanism at a temperature of 5° C., with only about 56% depression in the morning hours and about 78% depression during the evening hours. More recently, temperature-independence has been reported for the daily rhythm of emergence of the fly, Drosophila (Pittendrigh, 1954).

The persistent daily rhythms of many animals have been shown to continue with their accurately regulated 24-hour cycles even though the phases of the cycles have been shifted experimentally so that they no longer bear their normal relationships to the external day-night cycles. These cycles may be shifted experimentally either by exposing the animals to altered times of light and darkness in 24-hour cycles, or by subjecting them to various periods of refrigeration. The shifted rhythms typically display no tendency to drift back to their normal relationships to the external physical ones.

Persisting primary lunar or tidal cycles have also been reported. The literature on these is surveyed briefly by Brown, Fingerman, Sandeen and Webb (1953), who described a persisting tidal rhythm of color change of the fiddler crab. The crabs, which simultaneously possessed persisting 24-hour cycles, showed in con-

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[1] These studies were aided by contracts between the Office of Naval Research, Department of Navy, and Northwestern University, NONR 122803, and the Marine Biological Laboratory, NONR 09703.

stant darkness semilunar rhythms which appeared to result from the periodic reinforcement of one of these rhythms by the other. Temperature-independence of the tidal rhythms was demonstrated by Brown, Bennett, Webb and Sandeen (1954). The tidal rhythm, like the daily one, could be shifted by illumination changes and thereafter the rhythm would not drift away from the new setting. In fact, it was shown unequivocally that the phase relationships of the normal tidal cycles were determined by the times of the tides of each local beach, and these would be retained in constant conditions.

It has been assumed by many investigators that the rhythmic changes in constant conditions were regulated by an internal clock capable of measuring very accurately periods of 24 hours. The existence of such an internal clock was finally established definitely by Brown, Webb and Bennett (1955). These investigators found that fiddler crabs carried in darkness from Woods Hole, Massachusetts, to Berkeley, California, (*i.e.*, through 51° of longitude) by airplane continued to possess a daily rhythm of color change essentially in phase with that of crabs left under similar conditions in Woods Hole, as controls. The transported crabs clearly had been able to measure a reasonably precise 24-hour period even during a period when every conceivable factor whose frequency was determined by the rotation of the earth had been extended to a cycle of about 27.3 hours. There was, however, a suggestion from this experiment that the accuracy of this internal clock, under these conditions of uncontrolled temperature, might not be great enough to account for the astounding precision that would be required for the cycles not to get measurably out of their normal phase relationships with the external day-night cycles during several weeks or even months as has been observed frequently.

The research to be described was conducted in order to learn more about the nature of any daily and lunar rhythms in the rate of O_2-consumption with the view that some clue might be forthcoming as to the nature of the means whereby temperature-independence of the frequency of the cycles in organisms might have been achieved.

Materials and Methods

For the experiments to be described, the salamander, *Triturus viridescens,* and two species of fiddler crabs, *Uca pugnax* and *Uca pugilator*, were used. The experiments with *Triturus* were performed in Evanston, Illinois. The salamanders were shipped to Evanston from western Massachusetts where they had been collected. The experiments with *Uca* were performed at Woods Hole, Massachusetts, using crabs which were collected at Chapoquoit, on Buzzards Bay about 6 miles north of Woods Hole.

The crabs, each weighing about 3 grams, and *Triturus*, similarly weighing about 3 grams each, were placed in individual respirometers and their rates of O_2-consumption measured continuously in automatic, recording respirometers (Brown, 1954). This type of respirometer permits the animals to be maintained in essentially constant O_2-tension, and to be subjected continuously to the natural fluctuations in barometric pressure throughout the course of the experiments.

In its essentials the respirometer comprised a 50-ml. Soxhlet distilling flask possessing a rubber stopper bored part way through with a cork-borer. The

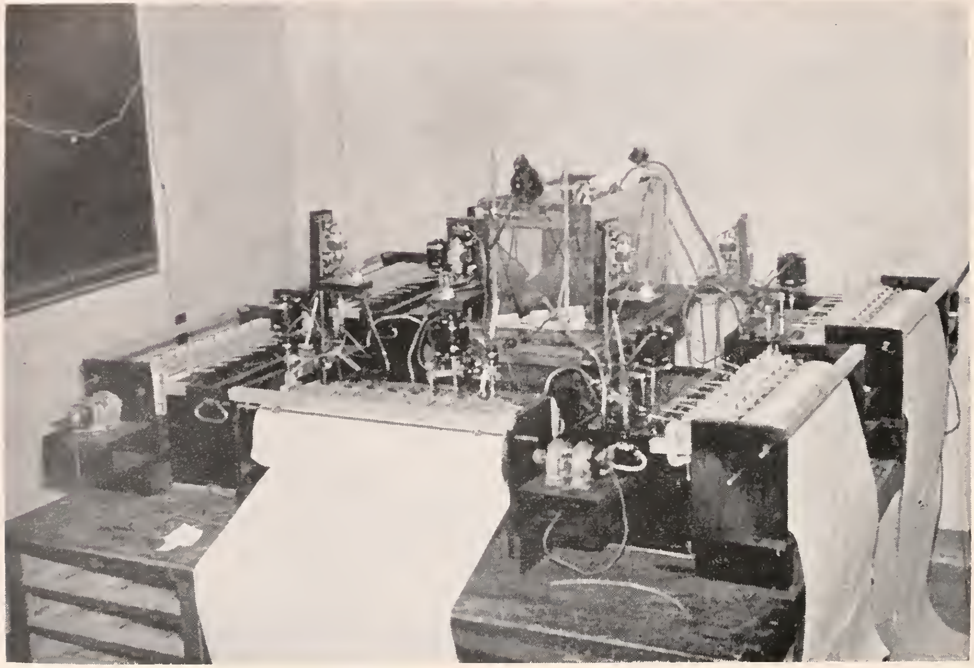

FIGURE 1. The arrangement of the baths, temperature-regulating equipment, and the recording respirometers operating from June 19 through August 30, 1954, at Woods Hole, Mass.

stopper was penetrated by a 27-gauge hypodermic needle with its point directed upwards through the stopper. To the lower side of the stopper were attached vessels for the CO_2-absorbent (KOH) and the ammonia-absorbent ($CuCl_2$) which were suspended into the flask. To the upper side of the stopper was sealed a plastic (Saran) collapsible sack which was, for all practical purposes, impermeable to O_2. In operation the animal was placed in the flask in a small amount of water; the sack was filled with oxygen; the vessels were filled with the absorbents, and the stopper set tightly in place. This ensemble, constituting a diver, was provided with just enough attached lead weights to sink it in water. It was then suspended by a fine wire in a constant temperature bath. The fine wire was attached to a very delicate spring scale with ink-writing points of such a character that the weight of the diver would be recorded continuously upon a moving strip of paper. The paper was drawn over a drum revolved by a telechron clock motor (Fig. 1).

It is evident that as the animals consumed oxygen, oxygen from the plastic sack would flow down the needle at a rate just adequate to compensate for that consumed by the organisms. For each milliliter of oxygen consumed, the diver would be expected to gain in weight by one gram. Since the sack had a capacity of about 50 ml., this was an adequate supply of oxygen to maintain the animals for upwards of a week without attention. In practice, however, the divers were opened at 3- to 4-day intervals, the O_2 and absorbents refreshed, and occasionally new animals were substituted at these times for those that had been in the respirometers for several days. Only extremely rarely did an animal die in a respirometer; in these cases the whole record for that organism, from the time the respirometers were last refreshed, was discarded.

The constant temperature baths in which the divers were suspended were maintained at 19.7° C. for the experiments in Evanston with *Triturus*, and 19.4° and 19.9° for the two baths which were used at Woods Hole for the experiments with *Uca*. In order to shield the divers from the agitated water of the bath, an aluminum container was submerged in the main bath, and it was in this aluminum container that the divers were suspended.

In all the experiments the animals were maintained in rooms protected from outdoor illumination. Incandescent sources gave a constant illumination of less than one ft. c. at the surface of the baths. The baths and aluminum container were painted flat black on their inner walls. Hence the organisms themselves were subjected to a much lower constant illumination which was not measured.

The experiments with *Triturus* were performed between May 12 and June 9, 1954. During this 29-day period there were continuously in operation eight respirometers containing *Triturus* and one respirometer treated in exactly the same manner except that no animal was added which was maintained as a control.

The studies of *Uca* were carried on without break between June 19 and August 31, 1954. Four crabs of each of the two species were run in each of two baths. In addition to the eight respirometers containing crabs, there was in each bath a diver lacking only the animal, run as a control.

In some earlier studies on O_2-consumption in snails and fiddler crabs (Sandeen, Stephens and Brown, 1954; Brown, Bennett and Webb, 1954) it had been assumed that the blank divers could be used as a proper means of correction for the concurrent fluctuations in barometric pressure. A more critical evaluation of the validity of this procedure has now clearly indicated that it is not a satisfactory method. In Figure 2 are depicted the variations in apparent mean respiration of eight *Uca pugnax*, over an arbitrarily selected 4-day period uncorrected for barometric-pressure effects. Superimposed upon this record, and plotted on the same scale are (1) the mean variations observed in five blank divers run concur-

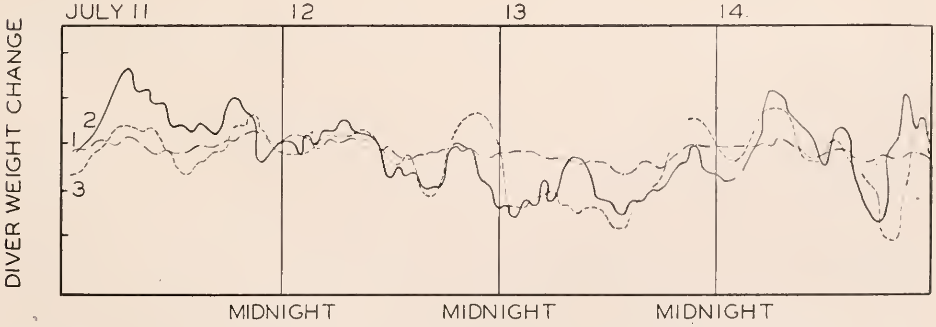

Figure 2. Plotted on the same scales are seen 1) the average hourly weight changes in the blank, control divers, 2) the average hourly weight changes for the crab-containing divers, and 3) the theoretically expected hourly changes in the blank divers on the basis of the actual barometric pressure changes, during an arbitrarily selected 4-day period.

rently, and (2) the expected variations in the blank divers calculated on the basis of the simultaneously recorded changes in actual barometric pressure. An analysis of 29 days of blank-diver data indicated that the blanks were responding, on the average, only about 27% of the theoretically expected extent. This error was finally traced to the mechanics of the spring scale. In order to decrease the sensitivity of the spring scale so that 3 to 4 days of uninterrupted recording of respiration of the animals could be obtained the spring scale had been adjusted so that the fluctuations in barometric pressure produced only relatively small movements in the long writing arm. In view of the virtual freedom of the blank divers from gaseous escape, the random fluctuations in barometric pressure produced only very small right and left excursions of the pen about a mean. Therefore, a large fraction of the expected range of pen variation was absorbed in a small freedom of movement in the pivotal bearings of the pen. The reality of this error was confirmed later by attaching blank divers to more sensitive scales. Under these latter circumstances the blanks approached more closely the changes expected in response to the actual pressure changes.

On the other hand, it can be seen that the respirometers containing the animals appeared to be behaving like more nearly perfect barographs. The total range of variation and the temporal relationships of most of the maxima and minima suggest this. Here it is evident that freedom of movement in the pivotal bearing of the scale writing arm was not of any influence. Due to a continuous utilization of O_2 by the animals, the pen was under essentially continuous, unidirectional tension. The pressure changes were translated into the record simply as spurious increases and decreases in rates of O_2-consumption.

In further confirmation of the assumption that the divers containing animals were actually behaving as essentially perfect barographs is a comparison of the average form of the daily variation in rates of gain in weight by the divers containing animals for 29 days, on the one hand, and the average form of the daily variation expected for blank divers on the basis of the measured variations in barometric pressure for the same 29-day period. This is seen for a 29-day period with the crabs, Figure 3A, and for the 29-day experimental period with *Triturus*, Figure 3B. It is seen clearly that not only are the phase relations essentially the same for the animal-containing respirometers and the blanks, but also the amplitude of the variation is closely similar. If the correction had been either 15 or 20% larger or smaller, rhythms simply correlated with barometric pressure would have been evident for all three species.

In the computations of the effect of barometric pressure, a diver volume of 100 ml. was always assumed and the corrections were calculated on the basis of an initial pressure of 30.00 inches Hg. Less than a 2% error was introduced in using this arbitrarily selected pressure instead of the actual barometric pressure. Assays of the divers revealed a mean volume of about 100 ml. for the 3- to 4-day respirometer runs. During this period there was a gradual volume reduction from about 110 ml. to about 90 ml. Therefore, about a 10% error was introduced through the use of this mean volume of 100 ml. The gradual drop in volume of the respirometers as the oxygen was utilized over 3 or 4 days would not contribute any rhythmic component of the frequencies being investigated. The general validity of this method is borne out by the gross good fit of the observed respirom-

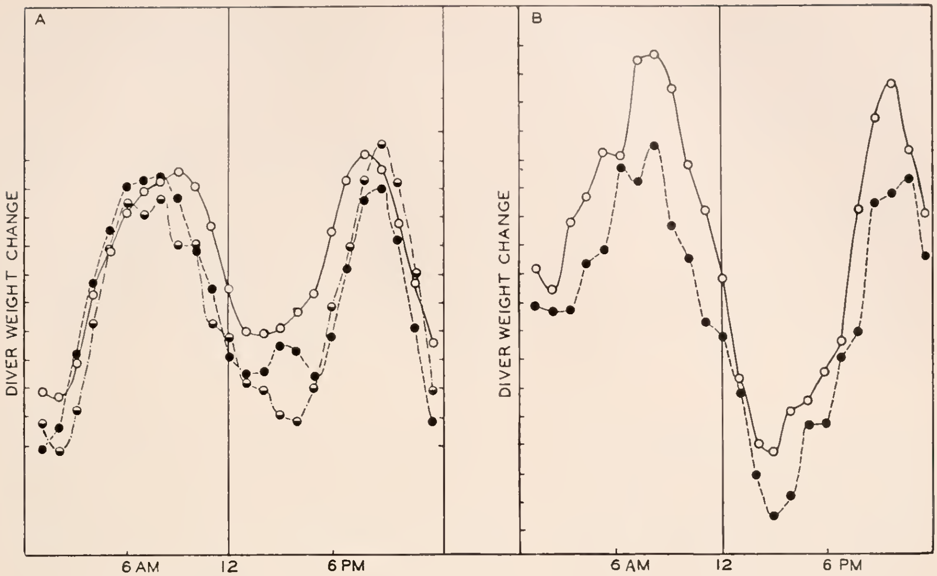

FIGURE 3. A. The mean daily variation in weight changes for a 29-day period of 1) divers containing *Uca pugnax* (open circles) and 2) divers containing *Uca pugilator* (solid circles) and 3) the theoretically expected 29-day, mean daily variation in the divers expected on the basis of the actual barometric pressure change (half open circles). B. The mean daily variation for 29 days of data with 1) divers containing *Triturus* (open circles) and 2) the mean daily diver variation expected on the basis of the pressure changes for the same period (solid circles). All are plotted on the same scale.

eter fluctuations and the calculated fluctuations based upon the concurrent pressure changes (Figs. 2 and 3).

However, since there is a clear mean daily rhythm of barometric pressure with an amplitude of the order of 0.05 inches Hg, it would be expected that there would be a mean daily rhythm imposed upon the volume of the respirometers and hence be reflected in the data corrected for the pressure on the basis of an initial pressure of 30.00 inches Hg used here. At those times of day when the barometric pressure is above the daily mean the corrections used would be too large, and for those times when the pressure was below the average, the corrections would be too small. The cyclic influences of the barometric pressure would be expected to be very slightly amplified, as a result of the correlated slight variation of the diver depth in the water bath and also through the operation of Henry's Law with regard to the water in the bottom of the respirometer chamber. A slight error is also introduced in correcting the volume as for a dry gas.

However, when one compares the form of the daily variation expected solely on the basis of the influence of the mean daily variations in barometric pressure on the respirometers, one finds in no case the form of the mean daily cycle of O_2-consumption to correspond. The rhythms appear to possess distinct species-specificity.

In the determination of the mean hourly correlation between the barometric pressure rate and direction of change and rate of the concurrent O_2-consumption, the values used in the calculation of each point included, indiscriminately, both overcorrected and undercorrected data due to any imposed barometric-pressure daily cycles. Therefore, the good correlations which were found are considered to be true biological responses.

It should also be pointed out that in setting up afresh the respirometers at three- to four-day intervals, the respirometers were closed a number of times in each 15-day or 30-day series, and in a random manner with respect to the actual barometric pressures at the times of change.

As a consequence of the foregoing considerations, the hourly respirometer data for the salamanders and crabs were corrected for the calculated influences of barometric pressure changes on the divers, upon the basis of a mean diver-volume of 100 ml. and of a uniform barometric pressure of 30.00 inches Hg.

RESULTS AND THEIR ANALYSIS

The rates of O_2-consumption for *Triturus* for each hour of the day for the 29-day period, May 12 through June 9, 1954, were determined and averages obtained for the eight animals which were studied concurrently. The rate of respiration during the 29-day period varied through a range of several hundred per cent from lowest to highest values. There was no regular pattern of daily variation, or overt daily rhythm. Superficially the day by day variation appeared to be random.

The rates of respiration for *Uca pugilator* for every hour of the period June 19 through August 30, 1954 were determined as were also those for *Uca pugnax* for the same period. As with *Triturus*, neither of the crabs exhibited any regular variations in rates of respiration from hour to hour or from day to day. There was not an overt daily rhythm of respiration. The variation during the period of observation yielded a range of the order of several hundred per cent from lowest to highest values, and appeared to be random in character.

The mean value of O_2-consumption for *Triturus* for the 29-day period was 28.12 ± 0.57. The mean value for *U. pugnax* for the 29-day period from July 27 through August 24 was 21.19 ± 0.43. The mean value for *U. pugilator* for the same period was 32.55 ± 0.53. If these values are multiplied by the conversion factor, 1.6, they become ml./Kg./hr. There seemed to be no evident correlation in forms of the day by day variations between *Triturus*, which was studied in May and June on the one hand, and the fiddler crabs which were studied in July and August, on the other, even when the days of data were synchronized as nearly as possible relative to the phases of the moon. Lack of correlation was confirmed when a determination of the coefficient of correlation of the hourly data for *U. pugnax* and *Triturus* with data brought as closely as possible into phase relative to sun and moon revealed a value of 0.0362 ± 0.038.

On the other hand, there appeared on superficial examination to be a significant correlation between the variations in the two species of fiddler crabs which were studied concurrently. A determination of the coefficient of correlation for the hourly values for the two species for the 29-day period July 27 through August 24 yielded a value of 0.401 ± 0.032. With the coefficient of correlation more than 12 times as large as its standard error, the existence of a positive correlation is beyond all reasonable doubt.

The random character of the hour by hour variations was emphasized when one determined the coefficient of correlation for the hourly values for *Uca pugnax* on day n with the values for *Uca pugilator* for day $n + 1$. An analysis of 29 days of data (June 19 through July 18) for such data dislocated by one day showed there to be no correlation (0.0117 ± 0.037). A low degree of correlation was seen when the values for day n were correlated with the values for the day $n + 1$ for a period of a month for the same species. This last, for *Uca pugnax*, provided a coefficient of correlation of 0.176 ± 0.034 and indicated a small tendency of the crabs to repeat on the next day the pattern exhibited on any given day.

On the other hand, when one attempted to correlate the hourly data for *Uca pugnax* and *pugilator* for one synodic month with the same species for the same hours of the days of the succeeding month, there was found for *pugnax* a value of 0.103 ± 0.032, and for *pugilator*, 0.007 ± 0.038. There is a suggestion of a correlation, even though a small one, in the hourly values of O_2-consumption for *pugnax* through days possessing comparable lunar relations. A similar suggestion was absent for *pugilator*.

In earlier work with fiddler crab rhythms of color change (Brown, Fingerman, Sandeen and Webb, 1953) and in respiration (Brown, Bennett and Webb, 1954) it was possible to resolve the variations into a complex of rhythms of different frequencies. These included ones of daily, primary lunar, semi-monthly and possibly monthly cycles. In order to determine if similar rhythms were present in these organisms using the more extensive and more critically corrected data, the data were first analyzed in such a manner that any existing daily cycle would be made evident. Since in a synodic monthly period every phase of a primary lunar

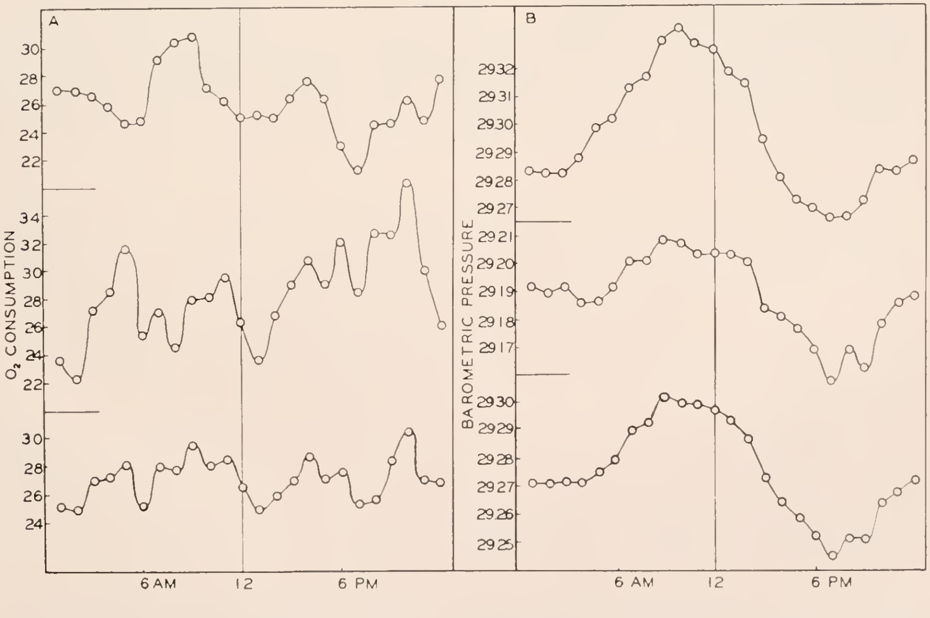

FIGURE 4. A. The mean daily variation in oxygen consumption for *Triturus* for 1) a 15-day period centered on full moon, 2) a 15-day period centered on new moon, and 3) a 29-day period. B. 1, 2, 3. The mean daily variations in barometric pressure for the two 15-day and 29-day periods in A, respectively.

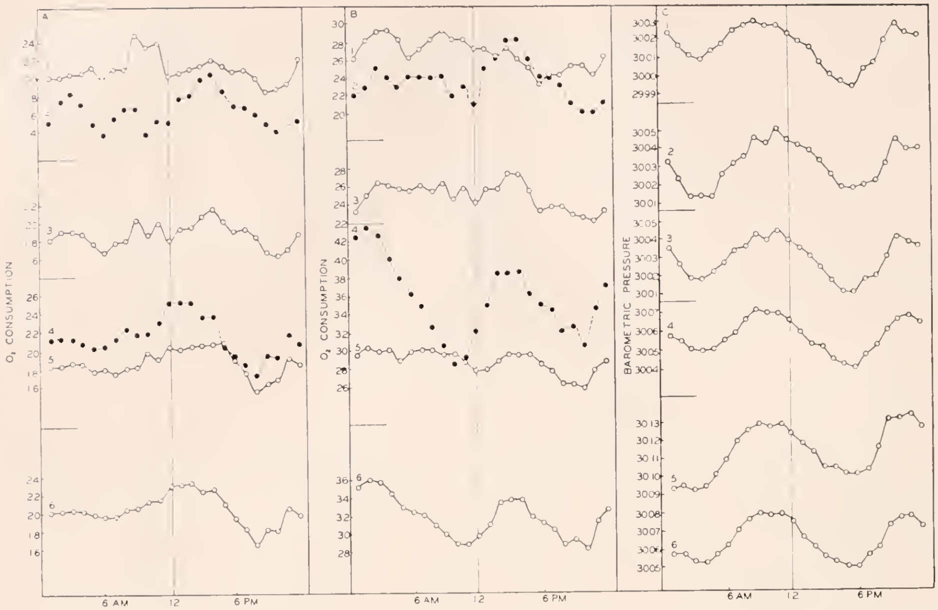

Figure 5. A. 1 and 5) The mean daily variation in oxygen consumption in *Uca pugnax*, for two consecutive new moon-centered 15-day periods. 2 and 4) The mean daily variation in oxygen consumption for two consecutive full moon-centered 15-day periods. 3 and 6) Two consecutive 29-day, mean daily variations in oxygen consumption. B. Comparable mean daily variations in oxygen consumption for the same periods as in A, but now for *Uca pugilator*. C. Mean daily barometric pressure variations for 1) the new moon- and 2) full moon-centered 15-day periods of the first month, and 3) the 29-day mean variation of the first month; 4) the new moon- and 5) the full moon-centered 15-day periods of the second month and 6) the 29-day mean variation of the second month.

cycle is present in the values for every hour of the day, the forms of the mean daily cycles were determined using 29 or 30 days of continuous data. In Figure 4A is found the mean daily variation for *Triturus* for the 29-day period, the mean daily cycle for each of two semilunar periods, and in Figure 4B the mean barometric pressure cycles for the same three periods. In Figure 5A, B and C are found comparable 15-day and 29-day mean daily cycles for *Uca pugnax* and *U. pugilator*, and the concurrent mean barometric pressure cycles for the periods June 23–July 21 and July 22–August 21.

The 29-day mean daily cycle of respiration for *Triturus* appears to possess four maxima, one in each of the four quarters of the day. The 29-day mean daily cycles for the two species of crabs appear to be rather similar to one another in their general form. There is a tendency for maxima to occur about 2 to 3 A.M. and about 2 to 4 P.M., with the lowest rate of the day occurring in the evening.

The daily rhythms for fortnights were calculated for periods with either new moon or full moon centered in the period. During 15-day periods from last quarter to first quarter the moon is above the horizon at noon and during the 15-day periods from first quarter to last quarter it is below the horizon at noon.

It is readily seen in the mean 15-day cycles that in every instance the form of the daily cycle differs between the two fortnights. In the fortnight straddling the full moon for *Triturus,* the lowest values of the day are in the late afternoon or early evening; the reverse is true of the fortnight straddling the new moon, the lowest values occurring before noon. For both species of fiddler crabs the mean daily cycle for the two fortnights straddling new moon exhibit only little change in rate through most of the day but show a clear minimal rate in the evening. For the fortnights straddling full moon, on the other hand, a much more prominent 2 to 4 P.M. peak in respiration becomes evident. In brief, there is clear suggestion in these fortnightly mean daily cycles of the existence of a persistent monthly cycle. The mean daily variations in barometric pressure also vary in every instance between the two 15-day periods. The morning rise in pressure is always greater for the fortnight straddling a full moon than for the fortnight straddling a new moon.

In order to determine the nature of any rhythmic differences through the month between the morning and afternoon values of respiration, the rate for the hours 5, 6, and 7 A.M., on the one hand, and 5, 6, and 7 P.M., on the other, were compared with one another and with both the mean hourly averages for the whole days and the barometric pressure means for the early morning and early evening hours. All are illustrated in Figure 6, 6A for the crabs and 6B for *Triturus.* In Figure 6A it is seen that there is a general tendency for the respiration of the crabs at the two times of day to fluctuate in the same manner as the daily mean values. While there is some correlation between the two species, there are also some conspicuous differences. There is not much evident correlation with barometric-pressure changes over the same period. A casual inspection suggests, however, that in the initial stages of a period of gradual increase in barometric pressure, there is a tendency for the O_2-consumption to decrease. Similarly, in the initial stages of a period of gradual fall in pressure, there tends to be an elevation in rate of respiration. This correlation becomes less, and it appears sometimes even to reverse itself after two or three days of a continuing rise or fall in barometric pressure. It is also to be observed that just as the morning values of barometric pressure tend to be higher than the afternoon ones during days of gradually declining pressure, and to be higher than the morning ones during days of gradually rising pressure, so do also the rates of respiration show comparable shifts in position, though not correlated with the barometric pressure itself.

In Figure 6B it is seen that what has been said for the crabs seems, in general, to hold for the salamander. Here, for the 5–6–7 P.M. period of the day there would appear to be almost an inverse correlation between pressure and the rate of O_2-consumption. This is less evident for the mean hourly values for the days, and virtually absent for the 5–6–7 A.M. values of respiration.

There is a suggestion for all three species of a tendency for the 5–6–7 P.M. values to possess maxima at about two-week intervals. The maxima tend to occur for these hours at the times of first and third quarters of the moon for the crabs and new and full moon for the salamander.

The data for the three species were next analyzed to learn what might be the general form of any existing primary lunar cycle of respiration. In order to differentiate a primary lunar cycle it was necessary to randomize the daily cycle. To accomplish this it was necessary to utilize a period of 29 or 30 days of continuous

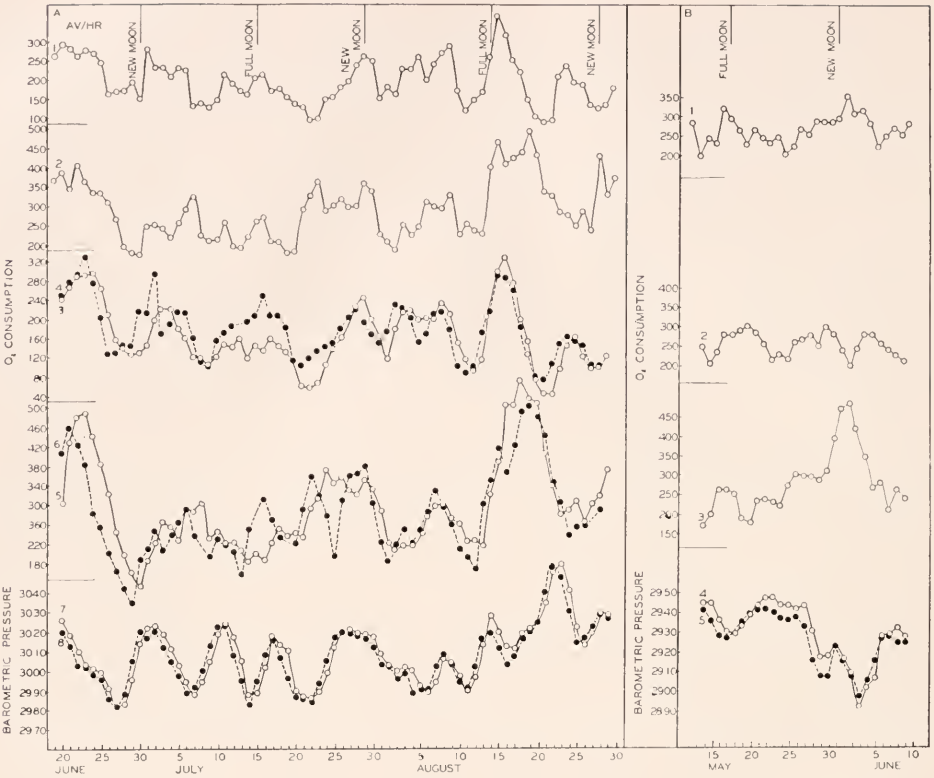

FIGURE 6. A. 1 and 2) Fluctuations in the mean daily rates of O_2-consumption of *Uca pugnax* and *Uca pugilator*, respectively, with time. 3 and 4) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. mean rates of O_2-consumption, respectively, of *Uca pugnax*. 5 and 6) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. mean rates, respectively, of O_2-consumption in *Uca pugilator*. 7 and 8) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M., respectively, barometric pressures. B. 1) Fluctuations in the mean daily rate of O_2-consumption of *Triturus* with time. 2 and 3) Fluctuations in the 3-day, sliding averages of O_2-consumption of *Triturus* for the 5–6–7 A.M. and 5–6–7 P.M. hours, respectively. 4 and 5) Fluctuations in, respectively, the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. values for barometric pressure.

data for each of the organisms. The 24 hourly values for the first day were arranged in a horizontal row. The data for the second day were placed, hour by hour, beneath those of the first day. The hourly values for the third, fourth, fifth, and sixth days were each shifted one hour to the left of the daily values lying just above each of them. Thus, the column under the noon values for the first two days would have the 1 P.M. value for the third day, the 2 P.M. value for the fourth, the 3 P.M. value for the fifth, and the 4 P.M. value for the sixth. The remaining 23 to 24 days of data were used in a comparable manner, continuing the shifting of the data to the left at the rate of five hours for every six days, or in other words, at the average rate of 50 minutes a day. After the 29 days of data have been used fully it is evident that a primary solar cycle has just scanned the 24 vertical columns beneath the values for the first day. The triangular block of data which no longer lies underneath the first day of data is then moved as a block to fill in the triangular void beneath the initial day. There are now 24 columns of 29 or 30 values in which any cycles of primary lunar frequency are now closely synchronized and there has been a neutralization of the daily cycle.

In Figure 7A are to be found mean primary lunar cycles for *Triturus*, and for the two species of crabs. In Figure 7B are the corresponding cycles of barometric pressure change calculated in the same manner from barographic data. It is evident that all three animals possess a cycle of variation in O_2-consumption of lunar-day length. In the figure all of the graphs have been synchronized with respect to the actual lunar phases, and the times of lunar zenith and nadir relative to them are indicated. Although the forms of the mean cycles vary not only with the species but to some extent even with the particular months for which they were determined, every one of them also exhibits an approximate lunar tidal cycle of about 12½ hours, with a minimum occurring 2 to 6 hours before lunar zenith and about the same interval before nadir.

The mean variations of lunar frequency for the barometric pressure appear somewhat irregular, although there is some suggestion of two tidal cycles in the lunar day in these data as well. The uniformity in the lunar cycles of the three organisms, however, is far more evident and similar than are the cycles of the barometric pressure.

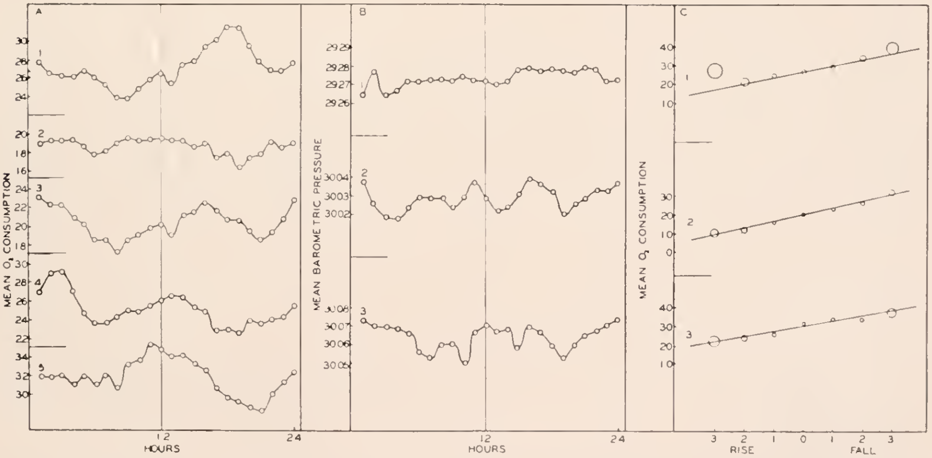

FIGURE 7. A. 1). The mean, 29-day, primary lunar variation of O_2-consumption in *Triturus*. 2 and 3) Two consecutive mean 29-day primary lunar cycles for *Uca pugnax*, and 4 and 5) two consecutive 29-day primary lunar cycles for *Uca pugilator*. Zenith is at 12 hours, nadir at 24. B. The mean variations in barometric pressure of primary lunar frequencies for 1) the 29-day period of the observations on *Triturus* and 2 and 3) the two consecutive, 29-day periods of study of the lunar cycles of *U. pugnax* and *U. pugilator*. C. 1) The relationship between the mean hourly rate of O_2-consumption in *Triturus* and the concurrent rate and direction of barometric pressure change, using all the hourly data of the 29-day period. 2) The same for *U. pugnax* using all the data of a two-month period. 3) The same for *U. pugilator* using all the data of a two-month period. The standard errors of the means are indicated by the diameters of the circles.

Since there was found to be a rather good positive correlation between the rates of O_2-consumption, hour by hour, for the two crabs which were studied concurrently, and either much less or no correlation when the daily values were correlated with the data just one day out of phase, it seemed suggestive that the rhythms of O_2-consumption were being regulated with respect to their frequency, at least, by hour-by-hour variations in some factor of the external physical environment. Consequently, the coefficients of correlation were determined for the hourly values of respiration for the three animals and the concurrent rate of change of barometric pressure ranging continuously from the maximum hourly fall to maximum hourly rise. The data for all three species gave highly significant negative coefficients. The value for *Triturus* was -0.457 ± 0.031; for *Uca pugilator* it was -0.375 ± 0.032 for one month and -0.320 ± 0.034 for the next; and for *Uca pugnax* it was -0.510 ± 0.027 for one month and -0.568 ± 0.027 for the next. In other words the rate of O_2-consumption appeared to be a clear function of the concurrent rate of pressure change; the greater the rate of pressure drop, the higher the rate of respiration, and the greater the rate of pressure rise, the lower the rate of respiration. Furthermore, in comparing the values for the two months for the two species of crabs, there would appear to be a species difference in the amount of the correlation.

Although the correlations are all quite high and unquestionably significant relative to the concurrent rates of barometric pressure change, when one determines the correlation with the same hourly rates of barometric pressure change for the preceding day (respiration, day n, vs. barometric pressure changes, day $n - 1$), values for *Uca pugnax* were -0.068 ± 0.037 for the period June 27–July 26, and -0.059 ± 0.037 for the period July 28–August 26. This appears to be a very low negative correlation, but its statistical significance is questionable. On the other hand, when a correlation was made for the same species with rates of barometric pressure change for the succeeding day (respiration, day n, vs. barometric pressure changes, day $n + 1$), a most unexpected result was obtained. For the period June 27–July 26 the coefficient was found to be -0.135 ± 0.037; for the period July 28–August 26 the value was -0.142 ± 0.037. Both of these values are of the order of twice the size of the correlation with the preceding day and each is statistically significant. It was obvious that the correlation of the barometric pressure change for the two pairs of days (n vs. $n - 1$ and n vs. $n + 1$) of the preceding periods would be essentially the same. When the hourly rates of barometric pressure on day n were correlated with the hourly rates on day $n + 1$ for the same period a value of 0.176 ± 0.036 was obtained. For barometric pressure changes, day n, correlated with barometric pressure changes, day $n + 2$, for the same period, it was 0.136 ± 0.037.

On the other hand, O_2-consumption in *Uca pugilator* for the hours of day n, whether correlated with the pressure changes during day $n + 1$ or day $n - 1$, gave equally low values, 0.002 ± 0.037 and 0.029 ± 0.037, respectively. If the difference seen for *Uca pugnax* should prove to be other than fortuitous and even be found for some other species, a possible conclusion would be that the crabs were responding to some environmental factor which itself in some manner is associated with an influence on the character of change in pressure for the next 24 hours or so.

The relationship between the rate and direction of barometric pressure change and the mean rate of respiration in the three species is illustrated in Figure 7C. Here are indicated both the mean rates and their standard errors. O_2-consumption values correlated with barometric pressure rates of change beyond .03 inches Hg/hr. were not plotted in these figures inasmuch as so few instances were available that their significance could not be established. For *Triturus*, a rise or fall of 0.01 inches Hg/hr. produces about a 12% change in respiration; for *Uca pugnax* a similar rate of change is correlated with about an 18% change, and for *Uca pugilator* with about a 9% change.

There appeared also to be a correlation between the absolute barometric pressure and the rate of respiration as was suggested in Figure 5A for the fiddler crabs. A calculation of the coefficient of correlation based upon the hourly values for *Uca pugnax* for the two-month period, June 27–August 25, and the concurrent barometric pressures provided a value of 0.057 ± 0.026. For *Uca pugilator* for the period, June 27–July 26, the corresponding value was 0.192 ± 0.036; and for *Triturus* for the period, May 12–June 9, it was − 0.149 ± 0.038. The last negative correlation had been suggested in Figure 5B. It is evident from these values that although significant correlations with barometric pressure are present for at least *Uca pugilator* and *Triturus*, these are of substantially lower degree than the correlations with the concurrent rates of barometric pressure change.

In less extensive experiments in which only 5 or 6 days of data were obtained a correlation was found between the rate of O_2-consumption and concurrent rate and direction of barometric pressure change for the three other invertebrates. For the sipunculid, *Phascolosoma gouldii*, the coefficient was − 0.480 ± 0.064. For the sea-cucumber, *Thyone briareus*, it was − 0.472 ± 0.069. The mud snail, *Nassa obsoleta*, gave a coefficient of − 0.264 ± 0.083.

Discussion

It would appear from this study that fiddler crabs and *Triturus* have daily, primary lunar and monthly rhythms of O_2-consumption which are not overt ones but which resemble the external rhythms in the physical environment in being statistical. Quite similar rhythms have also been found for O_2-consumption in three plants, the potato, carrot, and a seaweed, *Fucus* (Brown, Freeland and Ralph, 1955.) The daily and lunar rhythms in the plants resemble those of the animals reported here, even in some detail. The potato and *Fucus*, furthermore, showed essentially the same character of correlation with barometric pressure as found for these animals. The carrot, on the other hand, showed a reduction in rate of O_2-consumption whenever the barometric pressure was either rising or falling, with the degree of reduction linearly related to the rate of change.

These cycles of O_2-consumption show a good similarity with the rhythms of opening and closing of the shells of the oyster under constant conditions (Brown, 1954b). The activity of the oysters was recorded continuously for 45 days by simple, mechanical, automatic recorders. The oysters similarly showed no overt daily or tidal cycles, but only statistical ones. The forms of the mean daily cycles obtained with the oysters, and the variations of the daily cycles with the two halves of a synodic month, resembled those found in the work reported here on O_2-consumption in all three species. The oysters tended to be open more when barometric pressure was falling and to be open less when barometric pressure was rising. This general confirmation of the forms of the daily and lunar rhythms found in O_2-consumption obtained with the automatic-recording respirometers by results using simple, mechanically-recorded activity in the oyster, gives further support to the method of barometric pressure correction which was used in this work.

Any external factor, either barometric pressure or some factor which shows some degree of correlation with pressure which influences the rate of O_2-consumption in the organisms in the respirometers appears, therefore, also to influence the average number of minutes per hour that groups of oysters are open. It seems reasonable to presume that any factors which increase the rate of O_2-consumption in the oysters would, in consequence, cause the oysters to remain open a greater percentage of the time, and on this basis the results become consistent in the sign of the relationships.

These rhythms which are imposed upon the animals by the rhythmic external factor or factors appear not to be overt ones. Barometric pressure changes include a very large random component. Furthermore, for these variations to be resolved into rhythms of a clear daily character requires the integration of several days of barometric pressure change data. It is also true that the pattern of the daily changes in overt rhythms such as those of color change in the crabs (Brown, Fingerman, Sandeen and Webb, 1953), retinal pigment migration in shrimp (Webb and Brown, 1952), are usually quite different from that of the basic metabolic rhythm and, for that matter, from one another. There is some reason to believe that the overt rhythms are directly regulated by an internal clock, and that it is this internal clock which is reset by light and temperature. The imposed rhythm seems to be unmodified when the internal clock is reset. In two experiments with *Uca pugnax* in which the daily cycle of color change was essentially inverted by 12-hour periods of chilling, the correlation of the hourly values of O_2-consumption with those of unshifted controls gave coefficients of 0.389 ± 0.035 and 0.395 ± 0.031.

The evidence suggests, therefore, that the imposed and the internally regulated 24-hour cycles may come to bear any phase relations one to the other and then have no tendency to drift to any other relationships.

Although we still know little or nothing about any functional interrelations between the internal and the imposed daily rhythms, it is tempting to speculate that the imposed one in some manner contributes to the observed temperature-independence of the internal clock, at least over extended periods. A reasonably accurate daily internal clock would seem also to be a necessity in the resolution of the phases of the external rhythms which become evident only when several days of data are averaged. The daily clocks of the animals may well be a consequence of the cooperation of these two rhythms. Suggesting this is the often-observed variation in the times that the animals go into the night phase in their overt daily rhythm of color change. The variation about a mean may be more than a half hour from day to day, and yet there is no statistical drift in constant darkness even over months. This would find a reasonable explanation in terms of the internal clock being reset continuously by the rhythms of those factors which are responsible for the imposed clock.

This work provides no answer to the question as to what is the nature of the varying external force which is responsible for the imposed daily rhythm of O_2-consumption. Since the response correlated with the rate of change in barometric pressure is greater than the response correlated with the absolute level of pressure it appears to be a type of force to which there is some accommodation by the organism. Also the relatively low hourly correlation may be in part because even in superficially good correlations, *e.g.*, Triturus 5–6–7 P.M. the respiratory changes often anticipate barometric pressure changes by one or two days.

The force involved could be barometric pressure itself, or it could be any of a number of other factors which show some degree themselves of correlation with barometric pressure changes. One such other factor is cosmic radiation (Barnothy and Forro, 1939). It has been shown by Brown, Bennett and Ralph (1954), that fiddler crabs exhibit a measurable response in the state of their pigmentary system to alterations in the intensity of cosmic-ray showers obtained by placing lead plates over the animals. Had there been no correlation of the hourly changes in O_2-consumption with barometric pressure changes, then it would have been reasonably clear that such radiation was not normally involved in the regulation of the metabolic rhythms. The correlation with barometric pressure that has been found in this study leaves a role of such radiation as a distinct possibility, though quite obviously it does not, on the other hand, provide any positive evidence for such a role.

There is good likelihood, judging from the known simultaneous influences of such forces as light, temperature and tactile stimuli, that if these organisms possess the capacity to respond to one type of these relatively low-energy, or diffuse, types of environmental stimuli such as are implied by these results, they also possess the capacity to respond to a complex of them. Supporting such a multiple-factor view is the fact that the forms of the rhythms and their monthly variations appear to correlate to some extent with barometric pressure, but at the same time have large significant variations at some times of day and month that show little indication of any correlation with pressure.

Summary

1. Persistent rhythms of primary solar and primary lunar frequencies are described for O_2-consumption of two species of fiddler crabs, *Uca pugnax* and *Uca pugilator,* and for the salamander, *Triturus viridescens*.

2. These rhythms appear to be statistical rather than overt ones. The forms of the rhythms become apparent only upon the averaging of several days of data.

3. The form of the daily variation in O_2-consumption shows a monthly variation, the form of the rhythm for a two-week period straddling a new moon being different from the form for a two-week period straddling a full moon.

4. The hourly values in the rate of respiration show a significant correlation with the concurrent rate of barometric pressure change. In all three species, the rate of O_2-consumption increases in a direct relationship with the concurrent rate of barometric pressure fall and decreases in a direct relationship with the rate of pressure rise.

5. There appears, in some instances, to be a correlation with the absolute concurrent barometric pressure, but this is distinctly less. The correlation was a positive one for the two species of fiddler crabs and a negative one for the salamander.

6. It was demonstrated that O_2-consumption in all three species of animals displayed, therefore, imposed daily rhythms and in all probability, also imposed lunar rhythms.

7. The striking similarity of the forms and phase relations of these imposed daily and lunar rhythms of O_2-consumption to ones which are being described elsewhere for three species of plants, the alga, *Fucus*, and the carrot and potato, is

pointed out. The rhythms are also compared with very similar statistical solar and lunar rhythms of opening and closing of the shells recently described for the oyster.

8. There is a brief discussion of the environmental forces which might be involved, and speculation as to possible relationships between these imposed rhythms and the demonstrated endogenous rhythms in producing the temperature-independent overt rhythms observed in many processes in animals.

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PERSISTENT DAILY AND TIDAL RHYTHMS OF COLOR CHANGE IN CALLINECTES SAPIDUS[1]

MILTON FINGERMAN

Dept. of Zoology, Newcomb College, Tulane University, New Orleans 18, La.

The phenomenon of persistent biological rhythms has been reviewed by Welsh (1938), Park (1940), Kleitman (1949), Webb (1950), and Brown, Fingerman, Sandeen and Webb (1953). These investigators have described diurnal rhythms with a frequency of 24.0 hours, tidal rhythms with a frequency of 12.4 hours or 14.8 days, lunar rhythms with a frequency of 29.5 days, and annual rhythms.

The melanophore pigment of the fiddler crab, *Uca,* shows a striking diurnal rhythm of migration. In its natural environment *Uca* is light by night and dark by day. This rhythm persists in constant darkness in the laboratory for at least several weeks without loss of synchrony with solar day-night or decrease in amplitude (Brown and Webb, 1948). The frequency of this rhythm is not altered by temperatures between 6° and 26° C. It has been demonstrated, however, to be inhibited at temperatures below 6° C.

Brown, Fingerman, Sandeen and Webb (1953) described a persistent tidal rhythm of the pigment in the melanophores of *Uca pugnax* and confirmed the persistent diurnal rhythmicity of this pigment. This tidal rhythm is superimposed upon the diurnal rhythm and leads to a third type, a semilunar rhythm, in which the diurnal and tidal rhythms are in the same phases relative to one another only once each 14.8 days. The phases of the tidal rhythm of pigment migration bear a definite relationship to the time of low tide in the vicinity of the Marine Biological Laboratory, Woods Hole, Massachusetts where the *Uca* were collected. In the Woods Hole area the normal tidal situation is semidiurnal, two low tides and two high tides during each lunar day of 24.8 hours.

The Gulf of Mexico and its adjacent waters exhibit a primarily diurnal tidal situation of one low tide and one high tide per lunar day. This tidal picture provides an excellent contrast with the situation at Woods Hole. This investigation was undertaken to observe the phenomena of persistent daily and tidal rhythms of color change in a species living under the influence of diurnal tides.

MATERIALS AND METHODS

The animals observed were immature blue crabs, *Callinectes sapidus,* of 10 to 40 mm. carapace width which had been collected in Lake Pontchartrain, Louisiana, by Drs. R. M. Darnell and R. D. Suttkus. From the evening of the day they were collected until the observations were completed the crabs were kept in aquaria partially filled with 25% sea water in an air-conditioned darkroom. Temperature was maintained at about 16° C. and the water in the aquaria was changed every

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second day. Sex was not taken into account in selecting the animals for study. The crabs were exposed to light only during the periods of observation which lasted only a few minutes each hour.

The melanophores of all the crabs were observed in the darkroom by the use of a stereoscopic dissecting microscope and microscope light at twelve hourly intervals beginning at 8 A.M. and the degree of dispersion of the pigment within the melanophores determined. The only exceptions occurred on March 5 and 6 when the chromatophores were observed hourly throughout the night. The chromatophore index of Hogben and Slome (1931) was employed to stage the melanophores. In this system the most concentrated condition of the melanin is described as stage 1, the most dispersed as stage 5, and the intermediate conditions as stages 2, 3, and 4. The melanophores of the anterior aspect of a walking leg of each crab were staged

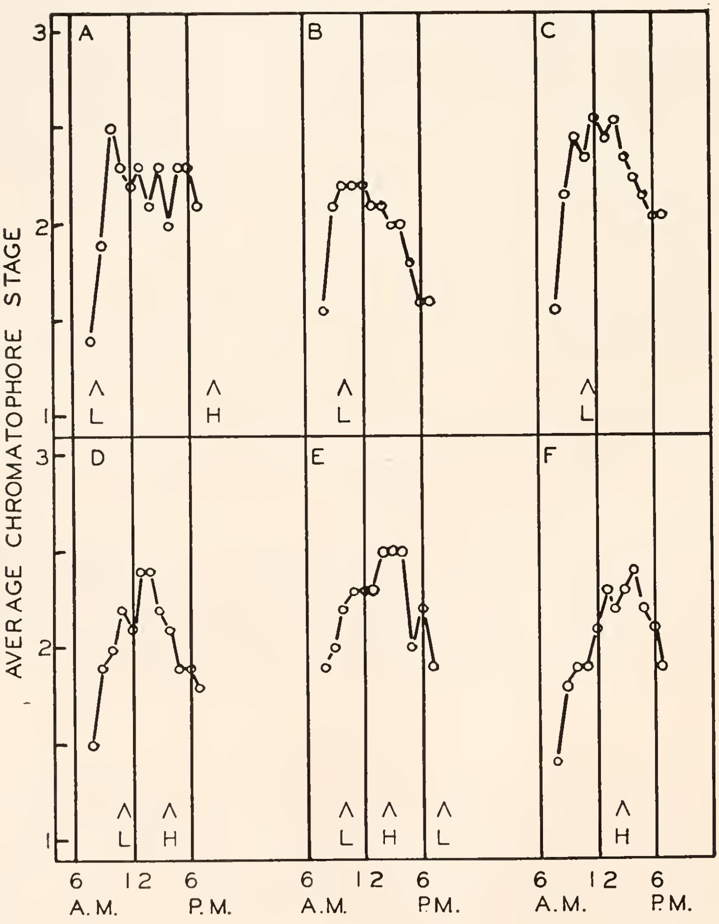

FIGURE 1. Changes in the black pigment of *Callinectes* in darkness. A is the record for Jan. 17, B for Feb. 19, C for Feb. 22, D for Feb. 24, E for Mar. 8, and F for Mar. 10.

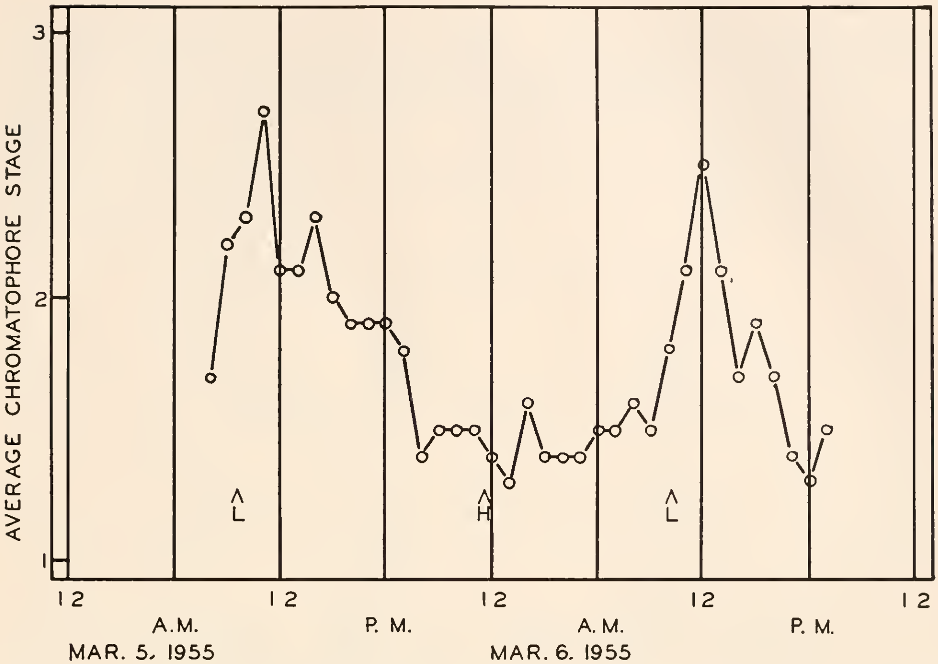

FIGURE 2. Changes in the degree of dispersion of the black pigment during a period in darkness.

and the average of the group was determined. The changes of coloration are not as striking in *Callinectes* as in *Uca*. In the former not only are there fewer melanophores per unit area on the legs but also the individual chromatophores of *Callinectes* do not exhibit the same high degree of branching that is found in *Uca* when the melanin is completely dispersed.

RESULTS AND DISCUSSION

A preliminary experiment (series A) performed on January 17th established the existence of a diurnal pigmentary rhythm (Fig. 1A). The crabs darkened by day and lightened by night. Twenty-nine animals were used.

A long period of observation (series B) occurred between February 19th and March 13th. These observations were begun with 52 animals. The number gradually diminished until only ten survived at the end of the observations. The hourly averages of the chromatophore stages for this series are presented in Table I. The second long period of observations (series C) was begun on March 5th and continued until March 13th. Twenty-one of the 28 *Callinectes* survived. The decrease in the number of animals had no effect upon the results. The reproducibility of the data obtained from the two long periods of observation (Fig. 3) indicates this. The hourly averages of the chromatophore stages for series C are presented in Table II. The times of low tide and high tide in Lake Pontchartrain on days observations of the chromatophores were made are presented in Table III.

In Figure 2 are presented the data obtained on March 5th and 6th from the *Callinectes* collected February 18th. In this figure the arrows below the curve indicate the times of low and high tide for these days in the original habitat of the animals. The pigment exhibited a diurnal dispersion and concentration; its maximal degree of dispersion occurred about noon. The major peak on March 5th occurred at 11 A.M. and a secondary peak was at 2 P.M. On March 6th both peaks shifted one hour to the right with the result that the principal maximum was at 12 noon and the secondary peak was at 3 P.M. This indicated that a tidal compo-

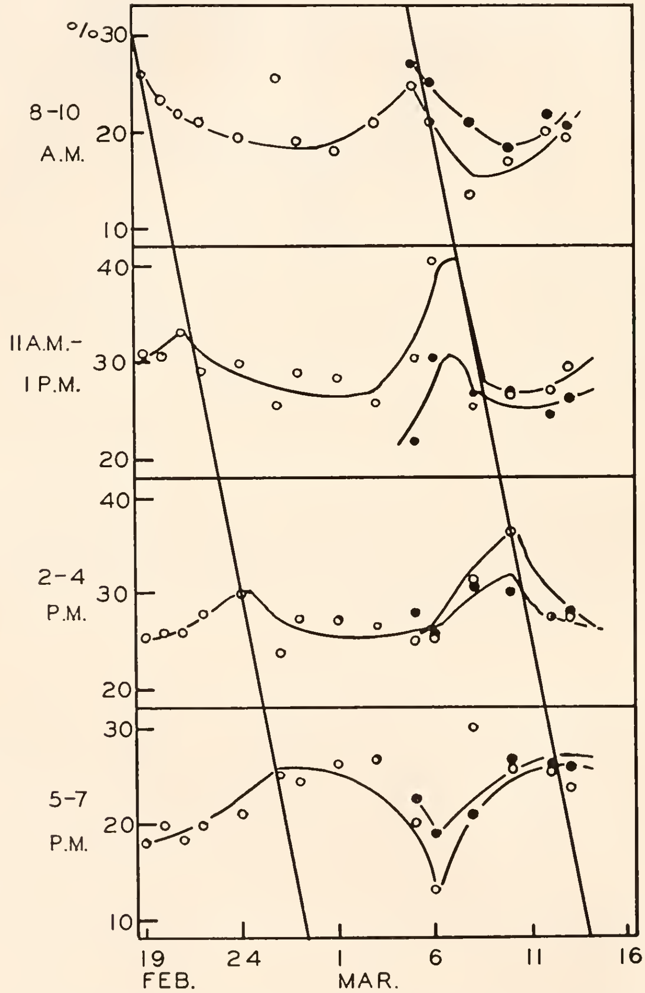

FIGURE 3. Relationship between the percentage of the daily melanin dispersion occurring at each of four periods during the day and the day of the month. Note the tidal maxima passing across the daily periods at the tidal rate of approximately fifty minutes per day. There is also a 14.5-day cycle, the interval between the diagonal lines. The circles represent animals collected Feb. 18, dots represent animals collected March 4.

TABLE I

The average melanophore index for each of the 12 daily measurements made during the first long period of observation (Series B)

Time	Date														
	February							March							
	19	20	21	22	24	26	27	1	3	5	6	8	10	12	13
8 A.M.	1.6	2.2	1.5	1.5	1.5	1.7	1.3	1.3	1.6	1.7	1.7	1.4	1.4	1.8	1.7
9	2.1	2.8	2.2	2.1	1.9	2.1	1.9	1.4	1.9	2.2	2.2	1.6	1.8	2.2	2.0
10	2.2	2.8	2.3	2.4	2.0	2.0	1.9	1.7	1.7	2.3	2.3	1.6	1.9	2.0	2.0
11	2.2	3.0	2.5	2.3	2.2	1.8	2.1	1.4	1.9	2.7	2.7	1.7	1.9	2.2	2.1
12 M.	2.2	3.3	2.5	2.5	2.1	2.0	2.1	1.7	1.9	2.1	2.1	2.0	2.1	2.4	2.4
1 P.M.	2.1	3.0	2.5	2.4	2.4	2.0	2.0	1.7	1.9	2.1	2.1	2.3	2.3	2.4	2.6
2	2.1	2.9	2.3	2.5	2.4	1.8	2.2	2.0	1.9	2.3	2.3	2.3	2.2	2.8	2.6
3	2.0	2.0	2.3	2.3	2.2	1.9	2.0	1.9	1.9	2.0	2.0	2.1	2.3	2.0	2.1
4	2.0	2.5	1.9	2.2	2.0	1.9	1.8	1.7	2.0	1.9	1.9	2.3	2.4	2.3	2.3
5	1.8	2.4	1.9	2.1	1.9	2.0	2.0	1.7	2.1	1.9	1.9	2.4	2.2	2.5	2.3
6	1.6	2.5	1.9	2.0	1.9	2.0	1.9	1.7	1.9	1.9	1.9	2.2	2.1	2.1	1.9
7	1.6	2.2	1.7	2.0	1.8	1.7	1.8	1.7	1.8	1.8	1.8	1.9	1.9	2.2	2.1

nent was superimposed upon the diurnal rhythm and was progressing across the scale at the rate of about one hour per day.

In Figure 1 are shown the data obtained on six other days with the times of low and high tide in Lake Pontchartrain indicated by the arrows. These days were selected to illustrate best the observed types of modification of the diurnal rhythm curve when the low or high tide was (a) early in the morning, (b) moving across noon, and (c) in the early evening.

TABLE II

The average melanophore index for each of the 12 daily measurements made during the second long period of observation (Series C)

Time	Date					
	March					
	5	6	8	10	12	13
8 A.M.	1.9	1.6	1.9	1.4	1.7	1.7
9	2.0	1.5	2.0	1.6	2.1	2.1
10	2.0	1.8	2.2	1.9	2.3	2.0
11	1.8	2.1	2.3	1.9	2.4	2.0
12 M.	1.8	2.5	2.3	1.9	2.1	2.3
1 P.M.	1.7	2.1	2.3	1.9	2.0	2.3
2	1.8	1.7	2.5	2.1	2.3	2.3
3	1.7	1.9	2.5	2.0	2.6	2.3
4	2.5	1.7	2.5	2.1	2.0	2.2
5	1.8	1.4	2.0	2.2	2.1	2.3
6	1.8	1.3	2.2	1.9	2.5	2.0
7	1.8	1.5	1.9	1.6	2.1	2.0

Table III

The times of low and high tides during the days observations were made. The times are corrected for Lake Pontchartrain where the Callinectes were collected

Date	Low tide	High tide
Jan. 17	8:14 A.M.	8:38 P.M.
Feb. 19	9:56 A.M.	11:38 P.M.
20	10:27 A.M.	—
21	10:50 A.M.	12:33 A.M.
22	11:04 A.M.	1:26 A.M.
24	10:48 A.M.	3:35 A.M.
	10:44 P.M.	3:40 P.M.
26	12:57 A.M.	4:14 P.M.
27	3:00 A.M.	4:58 P.M.
Mar. 1	5:53 A.M.	6.51 P.M.
3	9:18 A.M.	10:35 P.M.
5	9:35 A.M.	11:43 P.M.
6	10:05 A.M.	—
8	10:07 A.M.	2:37 A.M.
	8:10 P.M.	2:29 P.M.
10	11:26 P.M.	2:22 P.M.
12	12.50 A.M.	2.46 P.M.
13	2.18 A.M.	3.38 P.M.

On January 17th (Fig. 1A) low tide was at 8:14 A.M. and led to a maximal response at 10 A.M. High tide was at 8:38 P.M., so late in the day that it kept the chromatophore pigments semidispersed throughout the day and into the evening. There was also the suggestion of a minimum at about 4 P.M., tending to produce a bimodal curve. This bimodality is due to the absence of a tide near the middle of the day. Therefore, the diurnal rhythm alone was functioning to maintain a peak near noon. On February 19th (Fig. 1B) as the low tide moved to 9:56 A.M. the peak shifted and the maximum degree of pigment dispersion occurred at approximately 11 A.M. The high tide progressed until it occurred so late in the evening that it had no effect upon the diurnal rhythm. Therefore, the pigment gradually concentrated throughout the afternoon because there was no evening tide to keep it dispersed. On February 22nd and 24th (Fig. 1, C and D) the time of the tide had advanced approximately one to two hours and these curves are skewed to the right of noon. The data in Figure 2 for March 5th and 6th would fit between Figures 1B and 1C if they had been plotted on the same graph. On March 8th (Fig. 1E) the major tide was at 2:29 P.M. and the curve has its maximum at approximately 3 P.M. This curve is, therefore, a mirror image of Figure 1B; the pigment here gradually dispersed throughout most of the day rather than gradually becoming concentrated as in Figure 1B. On March 10th (Fig. 1F) the curve is skewed even more to the right. The maximum occurred at 4 P.M.

Evidently, as the time of the tide progressively becomes later in the day, a tidal component moves across the diurnal rhythm at the same rate that the tides are moving. In general, the tide exerts its maximal effect one to two hours after the time of the high or low tide.

The tides of the Gulf of Mexico and its adjacent waters are generally diurnal, there being one low and one high tide every lunar day. But, for approximately three days of every tidal cycle the tides tend to become semidiurnal; there are two low and two high tides per lunar day. This phenomenon occurs twice each lunar month when the moon is in the plane of the equator (Fig. 1, D and E). The data depicted in Figure 1D were obtained from observations of animals that were responding only to the time of low tide. It is obvious from the figure they continued to respond only to the time of low tide in spite of the appearance of a high tide later in the day. Furthermore, the response of the melanophores to a tide which occurred at that time of day did not lead to a curve with the shape of the one represented on February 24th (Fig. 1D). Three tides occurred during the hours of observation on March 8th (Fig. 1E), but the crabs responded only to the tide which occurred about 50 minutes later than on the previous day. A prediction based on the data of March 5th and 6th (Fig. 2) indicated that the peak which occurred at approximately 2 P.M. on March 8th (Fig. 1E) was to be expected. The two low tides which occurred at 10:07 A.M. and 8:10 P.M. could not have led to a curve with the shape of the one obtained on March 8th (Fig. 1E) because of the times of day at which they occurred.

The rhythmical tidal center in the animal is operating solely on the basis of tides that are spaced 12.4 hours apart, independent of whether they are both the same type of tide; and the shift to a semidiurnal tidal picture has no effect upon the center of tidal rhythmicity. This fact provides further evidence that these rhythms of *Callinectes* are endogenous and not directly influenced by the tides because the existence of a supernumerary tide has no influence upon the animals.

The *Uca* at Woods Hole, Massachusetts also show a response to tides spaced 12.4 hours apart (Brown, Fingerman, Sandeen and Webb, 1953). But the *Uca,* which were living in a permanently semidiurnal tidal situation, showed augmentation of the degree of dispersion of the melanin one to three hours after a low tide and not after a high tide. However, *Callinectes* exhibits a response not only to a low tide but also to a high tide which occurs for most of the tidal cycle 12.4 hours on the average after the low tide, which is the same interval of time that separates the two low tides at Woods Hole, Massachusetts.

To demonstrate the rate at which the tidal rhythm progressed over the diurnal rhythm, the following method of analysis was employed. The twelve periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M. and 5–7 P.M. The percentage of the total daily excursion that each three-hour period occupied was then calculated. This was done by summing the twelve average stages of the chromatophores which were obtained throughout the day and subtracting twelve from the total. If there had been no daily dispersion of the pigment, the sum of the indices would have been twelve. In like manner, the averages of the chromatophore indices for each three-hour period throughout the day were summed and three was subtracted from the daily total. The percentage of the total that each three-hour period occupied was then calculated. The data obtained in this fashion from series B and C have been used in the preparation of Figure 3.

The tidal maxima and minima travel across the scale at a rate closely approximating that at which a tidal cycle would be expected to progress (Fig. 3). This is illustrated by the two parallel diagonal lines drawn through the maxima at a distance of 14.5 days from one another. This is approximately the expected interval between days on which a low and a high tide will occur at the same time of day at any given location. Here again it is obvious that the animals are responding to both high and low tides; if they were responding to only one or the other, the maxima would be displaced from one another by approximately 29.5 days, which is the interval between days on which a low tide or a high tide occurs at the same time of day at any given location where the tides are diurnal.

The similarity of the results obtained with both groups of animals is striking (Fig. 3), the maxima and minima occurring at approximately the same day of the month in both groups. Or in other words, the animals which were in the laboratory darkroom for fourteen days prior to the collection of the next group of animals continued to maintain their tidal and diurnal rhythms in synchrony with those animals which were still in the field subject to the rhythmic variations of tide and light.

In Table IV are listed the daily amplitudes of the diurnal rhythm for series B and C. These values are the total of the average melanophore stages for the twelve daily measurements. There is no apparent increase in the amplitude of the rhythm with time in darkness. Brown, Fingerman, Sandeen and Webb (1953) showed that the amplitude of the diurnal rhythm of *Uca* gradually increased day by day until a maximum was reached in about ten days. The value for the second day of series B is much higher than ordinarily observed which probably is due to the fact that on this one day the air-conditioner failed and the darkroom was much warmer than on any other day. This indicates that the amplitudes of the rhythms are dependent upon temperature although their frequencies are independent of temperature.

TABLE IV

The amplitude of the diurnal rhythm of animals maintained in darkness. The amplitude is expressed as the total of the average stages for the 12 daily measurements of the melanophores

Date	Series B	Series C
Feb. 19	23.2	
20	32.5	
21	25.5	
22	26.3	
24	24.4	
26	22.9	
27	23.0	
Mar. 1	21.5	
3	22.5	
5	24.9	22.6
6	21.1	25.8
8	24.1	26.6
10	24.5	22.4
12	26.9	26.2
13	25.9	25.7

GENERAL DISCUSSION

Melanophore pigment rhythms of *Callinectes sapidus* have precise frequencies and must, therefore, be temperature-independent within the normal physiological range. If they were not temperature-independent they would have no adaptive significance to poikilothermic or heterothermic animals.

In one respect investigation of the pigmentary rhythms of *Callinectes* is more advantageous than with *Uca* because the amplitude of the diurnal pigment migration of *Callinectes* does not increase with time in darkness as it does in *Uca*. As a result, all phases of the tidal and diurnal rhythms exhibit a maximal expression throughout the entire period of the observations and are not obscured after a period of about ten days as they are in *Uca*.

The 12.4-hour frequency of the tidal rhythm in blue crabs is interesting because of the diurnal nature of the tides in its natural habitat. *Callinectes* responds in the same manner to high and low tides when these tides occur at the same time of day, whereas *Uca* responds only to low tides in the semidiurnal tidal habitat near Woods Hole, Massachusetts.

The evolution of tidal rhythms may have resulted from the existence of two events 12.4 hours apart to which the animals were exposed. The tidal rhythm may now be independent of the original events by virtue of an endogenous timing mechanism. In the evolution of precisely timed biological rhythms the nature of the two events which occurred at a set frequency may have been of secondary importance to the set frequency itself which in time became impressed upon the organism as a deep-seated phenomenon.

The interaction of the diurnal and tidal pigmentary rhythms in *Callinectes sapidus* is a basis for long term rhythms, just as found in *Uca,* because the tidal and diurnal rhythms come into synchrony only once every 14.8 days approximately, which is half a lunar cycle. If the *Callinectes* had been responding differently at times correlated with high and low tides, they would have been in an identical condition physiologically only once every lunar cycle.

The continued response of *Callinectes* to a diurnal tidal situation on days that the tides in its habitat become semidiurnal demonstrates that the tidal rhythm is a deep-seated phenomenon and is not easily altered by environmental conditions.

SUMMARY

1. The pigment in the melanophores of *Callinectes sapidus* displays an endogenous diurnal rhythm with a frequency of 24.0 hours. The pigment is in the dispersed state during the day and in the concentrated state at night.

2. The amplitude of the diurnal rhythm neither increases nor decreases under constant laboratory conditions.

3. Superimposed upon the diurnal rhythm is a tidal rhythm with a frequency of 12.4 hours. This rhythm is manifested by a supplementary dispersion of the melanin which occurs about fifty minutes later each day.

4. The tidal rhythm is most evident when the low or high tide is either in the morning or late afternoon. When either a low tide or a high tide occurs at these times, the diurnal rhythm curve is skewed to the left or right or tends to be bimodal, depending upon the times of high and low tides. There is no difference of response to low and high tides.

5. The tidal rhythm of *Callinectes* maintained under constant laboratory conditions has its phases bearing a definite relationship to the times of low tide and high tide in the native habitat of the crabs.

6. The *Callinectes* continue to exhibit their response to tides spaced 12.4 hours apart even on days that the usually diurnal tidal cycle of their original habitat becomes semidiurnal.

7. *Callinectes* also exhibits a semilunar rhythm. Only once every 14.8 days are the diurnal and tidal rhythms in the same phases relative to one another.

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MORPHOGENETIC MOVEMENTS OF NORMAL AND GASTRULA-ARRESTED HYBRID AMPHIBIAN TISSUES

JOHN R. GREGG AND DEANA KLEIN

Department of Zoology, Columbia University, New York 27, N. Y.

From a certain morphological point of view, some hybrid embryos behave like normal embryos treated appropriately with chemical inhibitors: they begin to gastrulate and then stop. Embryos belonging to the hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂, for example, stop gastrulating almost as soon as they begin (Moore, 1946). Externally, however, they look very much like ordinary *Rana pipiens* embryos that have been arrested at the same stage by exposure to 2,4-dinitrophenol or other metabolic poisons (Ornstein and Gregg, 1952). In fact, direct observation of blocked embryos generally does not yield evidence relevant to classifying genetical or chemical inhibitors according to their effects upon morphogenetic movements of different sorts—although the parts of normally gastrulating embryos severally undergo complexly interrelated displacements of different morphological kinds (see Nelsen's classification, 1953). Recently, however, Holtfreter (1944) has discovered that many normally-occurring gastrular movements can be modelled in isolation by movements that take place in suitably constructed explant systems; and application of his techniques to chemically treated tissues has already provided some warrant for the belief that gastrular movements are differentially retarded or suppressed by some metabolic poisons (Gregg and Ornstein, 1953). In the work about to be reported, we have extended Holtfreter's methods to study the capacities of gastrula-arrested hybrid tissues to undergo surrogate morphogenetic movements. On the basis of the results, we shall try to explain in morphological terms why hybrids of the kind studied fail to gastrulate.

METHODS

General

Appropriate explants were obtained by dissecting *Rana pipiens*, *Rana sylvatica* and *Rana pipiens* ♀ × *Rana sylvatica* ♂ Stage 10 (Shumway, 1940) embryos in Holtfreter's solution (often without bicarbonate, usually with 0.1 gm. sodium sulfadiazine per liter, at pH's ranging from 5.5 to 8.4). These were assembled into explant systems whose behavior was observed intermittently for periods up to 48 hours, at temperatures ranging from 9° to 25° C.

For our purposes, we shall say that the environmental conditions indicated constitute the set of *standard* conditions, since the morphological characteristics of the tissue movements that we have studied do not seem to be functionally related to environmental changes of the specified sorts.

For an account of explant systems more detailed than that given here, the reader is referred to the paper by Gregg and Ornstein (and also to that of Holtfreter). However, the systems discussed therein differ from some of those of present concern, and some brief descriptive and interpretative remarks are thus in order.

Type I. (Systems of this type not used in investigations now being reported.)

Type II. Exactly as described by Gregg and Ornstein. Rider consists of some presumptive notochord set uncoated side down on uncoated surface of yolk endoderm base. Endodermal part of rider expected to embed in base in movement (called "endodermal embedding in endoderm," or, briefly, "Em(en,en)") construed as homolog of bottle-cell formation. Mesodermal part of rider expected to elongate in its original meridional axis in movement (called "mesodermal stretching on endoderm," or, briefly, "St(m,en)") construed as homolog of convergent stretching.

Type III. (Systems of this type not used in investigations now being reported.)

Type IIIa. Systems of this kind like those of type III, except for omission of mesodermal component. Surface-coated ectodermal rider set uncoated side down on uncoated surface of yolk endoderm base. Rider expected to spread in sheet over base in movement (called "ectodermal spreading on endoderm," or, briefly, "Sp(ec,en)") construed as homolog of epibolic spreading.

Type IV. System consists solely of explanted Type II-system rider. Behavior expected: explant heals, becoming spherical; mesodermal portion elongates in movement (also called "mesodermal stretching on endoderm," or, briefly, "St′(m,en)"—the accent marks the difference, if any, from St(m,en)) construed as homolog of convergent stretching.

Presentation of data

We shall present our work in the manner of Gregg and Ornstein: the data will be summarized in a sequence of abbreviated expressions, interspersed with commentary. Because the systems dealt with here are of more complex origin than theirs, we shall have to complicate their abbreviatory conventions in detail, though not in principle.

The general abbreviatory form is:

$$M(g_t, g'_{t'})S:C;\ (k)n\mathrm{P};\ q\mathrm{N};\ r\mathrm{I}$$

where:

M is the sort of movement studied: stretching (St,St′), embedding (Em), spreading (Sp);

g and *g′* are the germ-layer types of the riders and the bases, respectively: endoderm (en), mesoderm (m), ectoderm (ec), (for Type IV systems, the endodermal portion will be regarded as base, the ectodermal portion as rider);

t and *t′* are the taxonomic types of the gastrulae supplying the riders and bases, respectively: *pipiens* (p), *sylvatica* (s), hybrid (h);

S is the type of explant system: II, IIIa, IV;

C is the set of environmental conditions: standard in every case;

k, n, q, and *r* are natural numbers; and

P is the set of positive results, N is the set of negative results, and I is the set of indeterminate results.

Thus, for example:

St(m_s,en_p)II: standard; (8); 7P; 1N; 0I

is read as asserting that of our attempts to obtain stretching of *sylvatica* mesoderm on *pipiens* endoderm, using Type II systems under standard conditions, 7 out of 8 were successful, 1 out of 8 was unsuccessful, and none yielded indeterminate results.

Slight departures from these conventions (see (23)–(28)) will be explained when the need arises.

Results

Morphogenetic movements of normal tissues

(2)–(4) merely confirm the results of Gregg and Ornstein in showing that *pipiens* tissues undergo the expected movements in explant systems:

(1) Em(en_p,en_p)II: standard; (7); 6P; 1N; 0I

(2) Sp(ec_p,en_p)IIIa: standard; (76); 67P; 6N; 3I

(3) St(m_p,en_p)II: standard; (22) 17P; 5N; 0I

(4) St′(m_p,en_p)IV: standard; (44); 38P; 1N; 5I

(5) shows that the tissues of *sylvatica* gastrulae, also, can exhibit some of these responses:

(5) Sp(ec_s,en_s)IIIa: standard; (4); 3P; 1N; 0I

Unfortunately, we do not have any direct information about Em(en_s,en_s), St(m_s,en_s) or St′(m_s,en_s), although results with interspecific tissue combinations ((7)–(12), below) strongly suggest that these movements can be made to occur unorthodoxly, and that *sylvatica* tissues do not differ from *pipiens* tissues in their ability to exhibit these movements (note (10), however).

Explant systems whose components derive from embryos belonging to different species behave, nevertheless, in generally normal fashion:

(7) Em(en_p,en_s)II: standard; (4); 4P; 0N; 0I

(8) Em(en_s,en_p)II: standard; (8); 7P; 1N; 0I

(9) Sp(en_p,en_s)IIIa: standard; (4); 4P; 0N; 0I

(10) Sp(ec_s,en_p)IIIa: standard; (10); 4P; 4N; 2I

(11) St(m_p,en_s)II: standard; (5); 4P; 1N; 0I

(12) St(m_s,en_p)II: standard; (8); 7P; 1N; 0I

(We do not have information about St′(m,en) in Type IV systems with interspecific tissue combinations.) In general, (7)–(12) are confirmatory of Holtfreter's claim that embryonic tissue affinities are not species-specific; the exception, (10) suggests that *sylvatica* ectoderm does not spread well on *pipiens* endoderm but further observations will be required to settle this point definitely (see (22)).

Morphogenetic movements of hybrid tissues

Embryos belonging to the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ will begin to gastrulate—each embryo finally exhibits a dorsal lip and develops an abbreviated archenteron. Therefore, if movements of the sort Em(en,en) are homologous with certain of those which accompany dorsal lip inauguration (*i.e.,* bottle-cell formation in the gray crescent area), then one would expect hybrid tissues to exhibit Em(en,en). This expectation is well-founded:

(13) Em(en$_h$,en$_h$)II: standard; (16); 16P; 0N; 0I

As a matter of fact, hybrid presumptive head endoderm embeds also in normal yolk endoderm:

(14) Em(en$_h$,en$_p$)II: standard; (9); 9P; 0N; 0I

(15) Em(en$_h$,en$_s$)II: standard; (5); 5P; 0N; 0I

and hybrid yolk endoderm provides a substratum in which normal presumptive head endoderm can embed:

(16) Em(en$_p$,en$_h$)II: standard; (4); 4P; 0N; 0I

(17) Em(en$_s$,en$_h$)II: standard; (8); 8P; 0N; 0I

Thus, insofar as Em(en,en) is concerned, hybrid tissues and normal tissues seem to behave alike.

But hybrid embryos only rarely gastrulate past Stage 10, and it is reasonable, therefore, to expect hybrid tissues to fail in respect to some of the other gastrular movements that it is possible to model in explant systems.

The evidence with respect to movements of the kind Sp(ec,en) is somewhat complex. Hybrid ventral ectoderm spreads well on hybrid yolk endoderm:

(18) Sp(ec$_h$,en$_h$)IIIa: standard; (13); 11P; 0N; 2I

and also on normal yolk endoderm:

(19) Sp(ec$_h$,en$_p$)IIIa: standard; (8); 7P; 1N; 0I

(20) Sp(ec$_h$,en$_s$)IIIa: standard; (3); 3P; 0N; 0I

But hybrid yolk endoderm appears to be less well suited than normal yolk endoderm as a substratum upon which normal ventral ectoderm can spread:

(21) Sp(ec$_p$,en$_h$)IIIa: standard; (20); 12P; 8N; 0I

(22) Sp(ec$_s$,en$_h$)IIIa: standard; (8); 4P; 3N; 1I

Before accepting this interpretation of (21) and (22)—that the high frequency of negative results is indicative of some peculiarity of hybrid yolk endoderm in respect to movements of the sort Sp(ec,en)—the reader should recall that *sylvatica* ectoderm seems to spread with a relatively low frequency of success upon normal yolk endoderm, as well (10).

With respect to models of convergent elongation the situation is more clear-cut, for explanted hybrid presumptive notochord invariably fails to elongate, no matter what the endodermal substratum provided:

(23) St(m_h,en_h)II: standard; (15); 0P; 15N(spread); 0I

(24) St′(m_h,en_h)IV: standard; (26); 0P; 26N(spread); 0I

(25) St(m_h,en_p)II: standard; (9); 0P; 9N(spread); 0I

(26) St(m_h,en_s)II: standard; (5); 0P; 5N(spread); 0I

What does happen in experiments of the sorts whose results are reported in (23)–(26) is that *hybrid presumptive notochord behaves toward an endodermal substratum like ventral ectoderm and spreads over it* instead of elongating in the fashion typical of presumptive notochord from normal embryos. (This is the significance of the word "spread" inserted after the itemizations of negative results in (23)–(26).)

(25) and (26) suggest that, for the failure of Type II and Type IV systems to exhibit movements of the sorts St(m_h,en) and St′(m_h,en), blame will have to be shouldered by hybrid presumptive notochord, which fails to elongate even upon normal endodermal substrata. On the other hand, there is at least some evidence that hybrid yolk endoderm is not completely satisfactory as a substratum for normal mesodermal activities of this kind:

(27) St(m_p,en_h)II: standard; (4); 2P; 1N(spread); 1I

(28) St(m_s,en_h)II: standard; (8); 4P; 2N(spread); 2I

In fact, (27) and (28) suggest that the presence of hybrid endoderm as a substratum can even stimulate tissues to spread—*i.e., pipiens* and *sylvatica* presumptive notochord—that behave in quite a different manner when explanted upon normal yolk endoderm. But we view this interpretation of (27) and (28) with some misgivings. If one attempts to explant presumptive notochord from gastrulae that are well into Stage 10, it is always possible that one mistakenly obtains ectoderm (presumptive neural plate) instead of mesoderm because of the difficulty of locating presumptive areas in partly gastrulated embryos—especially in the dorsal lip region. Whenever possible, we have dissected gastrulae as soon as their dorsal lips were detectable, but it is at least conceivable—we cannot decide more definitely about this point—that some tissues that we thought were mesodermal were actually ectodermal and that the seemingly negative results in (27)–(28) are explainable on this basis. Further evidence is needed to settle this question.

Discussion

We believe that our results provide us with certain key data for a morphological explanation of the fact that hybrid embryos begin to gastrulate and then stop.

Our proposal is this. When embryos gastrulate normally, their presumptive notochords invaginate; and, whenever presumptive notochords invaginate, their cells converge medially (an unambiguous diagram is given in Barth's *Embryology*, p. 121, Fig. 6.14). Now, from Moore's description of hybrid embryos, it is clear that their head endoderms invaginate and come to underlie their presumptive notochords. We have shown ((23)–(26)) that whenever hybrid presumptive notochords are provided with endodermal substrata in explant systems, they spread like ectoderm instead of converging medially like normal presumptive notochords; and we assume that something similar happens in intact hybrid embryos—that after invagination of their head endoderms has provided their presumptive notochords with endodermal substrata, the tendency of their notochordal mesoderms to spread in all directions upon these substrata effectively overcomes whatever tendencies their mesoderms have to converge medially. It follows that hybrid embryos cannot gastrulate normally.

Of course, it may be the case that hybrid notochordal mesoderms have *no* tendencies to converge medially: this in itself might be a sufficient reason why hybrid embryos do not gastrulate normally. To obtain information on this point, we explanted, without head endoderm, 9 hybrid presumptive notochords, all of which remained roughly spherical after healing, and 7 *pipiens* presumptive notochords, 5 of which thereupon exhibited typical notochordal elongation. Thus, our first explanation may explicate only one of several morphological reasons why hybrid embryos fail to execute normal gastrular movements. In any case, hybrid presumptive notochords are decidedly abnormal in their capacities to perform morphogenetic movements.

Summary

1. Explant systems have been constructed to test the abilities of isolated normal (*Rana pipiens*, *Rana sylvatica*) and hybrid (*R. pipiens* ♀ × *R. sylvatica* ♂) gastrular tissues to undergo activities surrogative of certain morphogenetic movements occurring in gastrulating embryos.

2. Evidence has been obtained to show that hybrid ventral ectoderm can carry out normal morphogenetic movements. On the other hand, there is some indication (see (10), (21), (22), (27) and (28)) that hybrid yolk endoderm is abnormal; and there is definite evidence ((23)–(26)) to show that the behavior of hybrid presumptive notochord is deviant from that of normal presumptive notochord.

3. Some tentative explanations are offered in morphological terms for the failure of hybrid embryos to gastrulate past Stage 10.

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CHANGES IN THE PROTOPLASM DURING MATURATION[1]

L. V. HEILBRUNN AND WALTER L. WILSON

Dept. of Zoology, University of Pennsylvania, Philadelphia 4, Pa., Dept. of Physiology and Biophysics, College of Medicine, University of Vermont, Burlington, Vermont, and the Marine Biological Laboratory, Woods Hole, Mass.

In recent years there has been a great interest in the physiology of cell division, and in many laboratories in various parts of the world investigators have attempted in so far as possible to obtain data that would help to elucidate the series of events that occur when a cell divides. From the standpoint of a physiologist, one of the most essential problems is the question as to the nature of the stimulus that induces a cell to begin the process of division. In order to find an answer to this question, a physiological attack is necessary; for no amount of morphological information, or morphological information combined with chemical recognition of morphological entities, can presumably offer much help. Certainly it has not been of much help thus far. What is needed is information as to which agents will cause cells to divide, which agents will prevent them from dividing, and why these agents act as they do. For this type of study marine egg material is ideal.

In our earlier work, we have been able to show that all the agents that induce marine eggs to divide cause a clotting of the protoplasm, a clotting typically inspired by the release of calcium from the cortex of the cell. The released calcium activates an enzyme system which is at the same time both a protease and a clotting enzyme. For a discussion of this earlier work, see Heilbrunn (1952a, 1952b), Heilbrunn, Chaet, Dunn and Wilson (1954), Heilbrunn and Wilson (1955), and Heilbrunn (1955). It is doubtless true that anything that causes a sea urchin egg or an egg of the worm Chaetopterus to undergo the first cleavage division, first causes a protoplasmic gelation, the so-called mitotic gelation. This is true both for the natural stimulus of fertilization and also for artificial stimuli of one sort or another.

But let us consider the case of immature eggs. These, too, following a proper stimulus (or the release of an inhibition), begin a division process. This process may involve only the splitting off from the large egg of a tiny cell, but the process is a division process nevertheless. And in an immature egg, with a large nucleus or germinal vesicle, the stimulus to divide is followed by a liquefaction of the protoplasm. How can this apparent contradiction be explained on the basis of our theory?

This was the problem we set for ourselves. It might be thought that anything which caused the breakdown of the germinal vesicle would release some substance which would liquefy the protoplasm. But there is also another possibility. If the calcium that is released as a result of stimulation does indeed activate an enzyme which is both a clotting enzyme and a protease, then such an enzyme might cause either a clotting of the protoplasm or a liquefaction—or it might indeed cause both.

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This idea appealed to us. In order to test it, we decided to determine whether the liquefaction of the protoplasm, which occurs when the maturation process begins, precedes or follows the breakdown of the germinal vesicle. If the liquefaction precedes germinal vesicle breakdown, then it could scarcely be held that the liquefaction was due to some substance released into the cytoplasm from the germinal vesicle nucleus. Also there might be a possibility that prior to the liquefaction there might be a mitotic gelation; although to tell the truth, when we began our investigation, we had small hope of obtaining such a result.

The viscosity of the protoplasm of the immature Chaetopterus eggs was determined by centrifuging pieces of egg-containing parapodia in a hand centrifuge of the type used in earlier studies from our laboratory. In order to make the tests rapidly, the handle of the centrifuge was turned once every second. This gave a force approximately 9,000 times gravity. Then at various time intervals after the eggs had entered sea water, the viscosity was measured in the same way. At first the results were not very uniform because of the fact that when the eggs were cut into sea water, some of the eggs came into contact with the sea water before others. Accordingly, it was found advisable to cut the worms on a piece of cheese-cloth, and then stir up the eggs that had emerged on to the cheese-cloth in sea water.

Figure 1 shows the results obtained at 21° C. The viscosity values are given as four times the number of seconds required to show zoning of the eggs. The reason why the number of seconds was multiplied by four is that earlier studies of the viscosity of the protoplasm of Chaetopterus eggs were made by turning the handle of the centrifuge once every two seconds. This gives a centrifugal force one-fourth that developed when the handle of the centrifuge is turned once every second. It might be thought that the protoplasm of immature eggs was thixotropic and that therefore values for the viscosity would vary with the shearing force. However, a series of tests at different centrifugal speeds, that is to say, at different shearing forces, showed that if the protoplasm is thixotropic at all, the thixotropy is slight, and that viscosity values obtained at different shearing forces are approximately the same.

From Figure 1 it is clear that the viscosity of the immature egg doubles within a half minute after the egg enters sea water. Following this, the viscosity drops rapidly, so that by the time the egg has been in sea water for just one minute, the viscosity has returned to the value it had when the egg was in the parapodium. By the fifth or sixth minute after entrance of the egg into sea water, the viscosity of the egg protoplasm has dropped so low that only three seconds of turning the centrifuge at one turn of the handle per second are necessary to produce sufficient movement of the granules to show zones. This represents a value for the viscosity of about 12, although, of course, in view of the fact that the number of seconds of turning required was so small at this point, no great accuracy was possible. Thus if anything more than two seconds were required to shift the granules, the value was recorded as three. In earlier work (Heilbrunn and Wilson, 1948), the viscosity of the unfertilized egg after maturation was completed was found to be about 11, so that we can assume that after the fifth or sixth minute of immersion in sea water, any further decrease in the viscosity of the protoplasm is slight. At 21° C., the germinal vesicle breaks down at the seventh minute, so that at this temperature the liquefaction of the protoplasm certainly precedes the breakdown of the germinal vesicle.

Hence we can assume that the liquefaction of the protoplasm that occurs in the Chaetopterus egg is not due to some substance liberated from the nucleus, for it occurs before the nucleus shows any signs of breaking down. Our results thus indicate that the liquefying effect is due to the protease described by Goldstein (1953). According to Goldstein, as soon as the Chaetopterus egg enters sea water, a proteolytic enzyme system is activated by the entrance of calcium into the cell interior. Goldstein believes that this protease is responsible for the breakdown of the germinal vesicle; our data seem to show that it also causes a liquefaction of the protoplasm. But before this liquefaction occurs, the protoplasm shows a very brief clotting reaction and this can be interpreted as constituting the mitotic gelation. We believe that both the gelation and the subsequent liquefaction can be caused by the same enzyme, for it is well known that calcium-activated proteases can act to produce both clotting and liquefaction. Indeed, thrombin itself is both a clotting and a proteolytic enzyme. For further discussion, see Heilbrunn (1955).

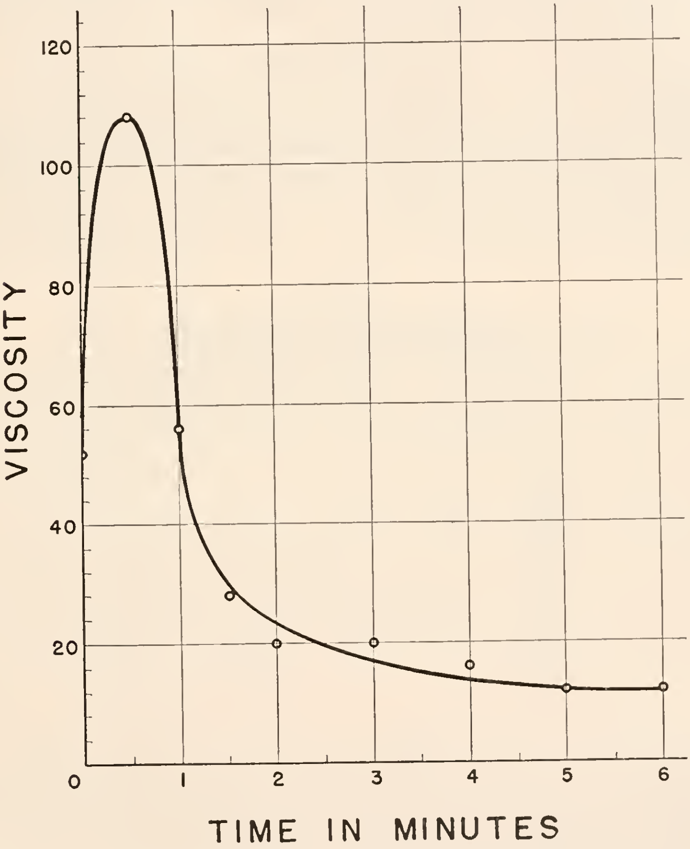

FIGURE 1. The viscosity of the protoplasm of the Chaetopterus egg at various intervals of time after the egg has entered sea water.

The curve shown in Figure 1 represents but a single set of data. It is supported by other data of the same sort obtained in other experiments at 21° C. Moreover, we did series of tests at various temperatures other than 21°. At temperatures from 6° to 24° C., it was always possible to show that the protoplasm became more fluid before the germinal vesicle broke down. At temperatures from 27° to 40° C., the germinal vesicle broke down very rapidly—in three to four minutes—and it was scarcely possible to observe any decrease in viscosity before the germinal vesicle broke down. At the lower temperatures, there seemed to be evidence of a rise in viscosity before the drop in viscosity occurred, but this was not followed as carefully as it was at 21° C.

Our primary interest was not in the effect of temperature on the rate of germinal vesicle breakdown. However, because the presence or absence of a germinal vesicle could rather easily be observed in the centrifuged eggs, it was possible for us to determine the time required for germinal vesicle breakdown at different temperatures. The data are not very remarkable, but they are presented here in the thought that they might be of interest to those who are concerned with the effect of temperature on various phases of mitosis. The results are given in Table I.

TABLE I

Time required for breakdown of the germinal vesicle of the Chaetopterus egg at various temperatures

Temperature	Time in minutes
6° C.	107
9	28
12	16
15	13
18	9
21	7
24	5
27	4
30	3
33	3
36	3
39	3
40	4

The question that we asked ourselves has certainly been answered. The initiation of maturation in the egg of Chaetopterus beyond any doubt causes the protoplasm of the egg to become more fluid even before the germinal vesicle breaks down. In preliminary experiments, we found this also to be true for the egg of the clam *Spisula solidissima*. In the Spisula egg, maturation does not begin until the egg is fertilized. Also, in the Chaetopterus egg, the liquefaction of the protoplasm is preceded by a transient increase in viscosity, indicative of a mitotic gelation. This mitotic gelation presumably is the precursor of the spindle of the first maturation division.

Our results indicate that when calcium is released into the protoplasm, it can cause a clotting reaction and it can also dissolve the clot or gel once it is formed. The double effect is due to the fact that calcium activates an enzyme which is both a clotting enzyme and an enzyme which has a proteolytic effect. There is an abundance of literature to indicate that such enzymes do exist. Indeed, clotting enzymes such as rennin and thrombin are also proteolytic, and proteolytic enzymes such as trypsin can act as clotting agents. The subject is discussed at some length by Heilbrunn (1955) and references to the literature are given there. The facts as presented here serve to explain what has been regarded as a rather anomalous effect of calcium on the protoplasm of the squid giant axon. In a study of this protoplasm, Hodgkin and Katz (1949) found that calcium ion exerted a dispersing or liquefying action and their work has been widely quoted. As a matter of fact, in the Hodgkin and Katz experiment, the protoplasm was probably gelled as a result of injury (see Heilbrunn, 1955). If calcium activates a proteolytic enzyme (which is also a clotting enzyme), calcium could either cause clotting or liquefaction.

SUMMARY

When an immature egg of the worm Chaetopterus enters sea water, the protoplasm first undergoes a transient increase in viscosity, and then rapidly becomes more fluid than it was originally. These effects are believed to be due to a calcium-activated proteolytic enzyme which is also a clotting enzyme. In the course of the investigation, data were obtained as to the effect of temperature on the rate of germinal vesicle breakdown.

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THE CONTROL OF NEUROSECRETION AND DIAPAUSE BY PHYSIOLOGICAL CHANGES IN THE BRAIN OF THE CECROPIA SILKWORM [1]

WILLIAM G. VAN DER KLOOT

The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

Twenty-six neurons control the growth and metamorphosis of the Cecropia silkworm. These are the neurosecretory cells of the brain—nerve cells specialized for endocrine function. In each brain the neurosecretory cells are gathered into four distinct groups which in favorable preparations can be seen as clusters of bluish-white spots when the living brain is exposed and viewed *in situ* through a dissecting microscope (Williams, 1948, 1952). The hormone secreted by these cells directs a series of events which result in the insect's growth. There is now ample evidence to show that the brain hormone acts primarily on a single organ, the prothoracic glands. By this tropic action the brain elicits the secretion of the prothoracic gland hormone which then circulates in the blood and reacts with the tissues to favor growth and metamorphosis (Williams, 1947, 1954).

In circumstances where the brain fails to secrete its hormone, growth comes to a standstill. Such an eventuality, as Williams (1946b) first showed, normally occurs in insects with a pupal diapause. Here the failure of the neurosecretory cells to secrete the brain hormone after pupation is the basis of the pupal diapause. Months later the neurosecretory cells release the brain hormone and development is resumed.

The return of activity to the brain's neurosecretory cells is known to depend on the temperature history of the pupa (Williams, 1946b, unpublished data). In pupae stored at room temperatures a minimum of five or six months must elapse before the neurosecretory cells secrete the hormone and thereby terminate the pupal diapause. Yet, when similar pupae are given preliminary exposure for two or three months to temperatures which mimic those of the normal winter environment, a remarkable change occurs in the functional capacity of the neurosecretory mechanism. The brain, after the low temperature treatment, is now able to secrete its hormone promptly at the room temperature. Presumably, the low temperature acts directly on the brain and its neurosecretory cells, for, as Williams (1946b) demonstrated, the implantation of a chilled brain into an unchilled pupa causes the latter promptly to begin adult development.

How the low temperature restores "competency" to the neurosecretory cells is a mystery. Indeed, *Rhodnius prolixus* is the only insect where the mechanism controlling the activity of the brain's neurosecretory cells has been analyzed. Wigglesworth (1933, 1934, 1940) showed that the release of brain hormone is triggered

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by the enormous distension of the insect's abdomen following a blood meal. Since transection of the ventral nerve cord blocks this effect, it was inferred that the neurosecretory cells are driven by nerve impulses arising in the abdominal proprioceptors.

Furthermore, in experiments on vertebrates (summarized by Scharrer and Scharrer, 1954) there is increasing evidence that hormone release by the hypothalamic neurosecretory cells is triggered by nerve impulses—a conclusion strengthened by the recent study of Schimazu *et al.* (1954). Additional information concerning the control of neurosecretory cells is obviously desirable, especially in view of the widespread occurrence of neurosecretory cells in both vertebrates and invertebrates. The brain of the Cecropia silkworm appeared to be a favorable object for further analysis, since by varying the environmental temperature the endocrine activity of the neurosecretory system can be regulated at the convenience of the investigator. The observations of Wigglesworth encouraged the use of neurophysiological techniques for relating nervous and neurosecretory activities.

Materials and Methods

1. *Experimental animals*

Caterpillars of the giant American silkworm, *Platysamia cecropia,* were reared under nets on wild cherry trees. After pupation the cocoons were promptly harvested, stored temporarily at room temperature for about one month, and then subdivided into groups and placed at 6°, 15°, or 25° C. A smaller number of experiments were performed on the closely related saturniids, *Telea polyphemus* and *Samia walkeri* (*cynthia*). Several studies were performed on the wax moth, *Galleria mellonella,* a non-diapausing lepidopteran which was reared in the laboratory at 33° C. on a mixture of Pablum and glycerol.

During adult development the stage of development can be estimated by reference to the detailed morphological time table of Schneiderman and Williams (1954). In the absence of detectable morphological changes during the interval between the transfer of diapausing pupae from 6° C. and the onset of adult development at 25° C., it was necessary to judge the status of the animals on a statistical basis. A homogeneous group of 116 pupae was transferred to 25° C. after chilling at 6° C. for 13 weeks. Of these, 93 were used for physical or chemical measurements before the visible onset of adult development. The absence of development was confirmed in all cases by dissection. The remaining 23 pupae were used to determine the time for the initiation of adult development; the median time was 9 days, with extremes of 7 and 16 days.

2. *Methods*

a. *Electrical activity.* A Grass P-3 preamplifier and a Dumont 304-A oscillograph were used. The recording electrodes were No. 50 gauge platinum wires, or electrolytically tapered stainless steel insect pins (Grundfest *et al.*, 1950; Burkhardt, 1954), or indium-filled glass microelectrodes (Dowben and Rose, 1953). A Grass S-4 stimulator and stimulus isolation unit were used for electrical stimulation. The stimulation was through tapered silver-silver chloride electrodes (Roeder, 1946). All electrodes were manipulated by micromanipulators similar to that described by Goldacre (1954), but made from "Lucite" rather than glass.

The integument overlying the brain (or ganglia of the ventral cord) was removed and the active electrode inserted into the nervous tissue by use of the manipulator. The indifferent electrode consisted of a stainless steel needle inserted through the integument at the anterior end of the animal and extending into the hemolymph for about two centimeters.

b. *Cholinesterase.* Two different techniques were used for the assay of cholinesterase (ChE); namely, a colorimetric and a manometric technique.

In the colorimetric analysis 4 brains or 9 to 12 ganglia were excised from animals previously anesthetized with carbon dioxide (Williams, 1946a). The tissues were rinsed in insect Ringer (Ephrussi and Beadle, 1936) and ground in a small, conical, glass homogenizer. The resulting homogenate was diluted by the addition of 3.6 ml. of a solution containing 0.56 M NaCl and 0.063 M KH_2PO_4; the pH of this solution had previously been adjusted to 8.0 by the addition of NaOH (Chadwick *et al.*, 1953). The homogenates, after dilution, were stored in the deep freeze at − 20° C. One to 42 days later the assay was performed. The homogenate was equilibrated with 25° C. in a water bath, and then 0.4 ml. of 0.15 M acetylcholine bromide (ACh, Eastman "reagent grade") was added to yield a final volume of 1.0 ml. per brain and a final concentration of 0.015 M ACh. Immediately after the addition of ACh, a one-ml. aliquot was removed, subdivided into 0.2-ml. samples, and assayed for ACh by the colorimetric method of Hestrin (1949). After incubation for a total of one hour, a second one-ml. aliquot was withdrawn and the ACh measured as just described. The non-enzymatic hydrolysis of ACh was determined by the use of a 4.0-ml. blank containing all reagents except the homogenate. The ACh content of one-ml. aliquots of the blank was measured immediately after the ACh addition and also after one hour's incubation. This correction was applied in the calculation of the amount of ACh enzymatically hydrolyzed per hour.

Ammon's (1933) method of ChE assay was used more generally since a homogenate hydrolyzing as little as 0.05 μM of ACh per hour could be detected when 5-ml. vessels were employed. This greater sensitivity permitted determinations on a single brain, or on 3 or 4 ganglia. In performing the analysis the homogenate of brain or ganglion was diluted to 0.9 ml. with a solution containing 0.56 M NaCl and 0.079 M $NaHCO_3$. The resulting suspension was transferred to 5-ml. Warburg vessels and the manometers gassed with 95 per cent nitrogen and 5 per cent carbon dioxide for five minutes. After a further 10 minutes of temperature equilibration at 25° C., 0.1 ml. of 0.15 M ACh was tipped in from the side-arm of each vessel. Shaking was begun immediately and the first reading was taken 5 minutes later and continued at 10-minute intervals for 30 minutes. The thermobarometer contained all components in the reaction mixture except the homogenate; it therefore corrected for the non-enzymatic hydrolysis of ACh. The results obtained by the colorimetric and manometric methods were indistinguishable and the data have been treated as a unit.

The results were computed on a per brain or per ganglion basis, rather than in terms of weight of tissue. To make possible rough comparisons of the data, groups of equivalent brains and ganglia were excised, gently blotted on filter paper, and weighed individually on a micro-torsion balance. The pupal brain weighed 1.22 ± 0.19 mg., and each ganglion, 0.19 ± 0.03 mg.

c. *Assay of cholinergic substances.* Brains or ganglia were dissected from unanesthetized animals and placed in one ml. of sea water containing 0.01 per cent eserine sulfate (Merck) and brought to pH 4 by the addition of dilute HCl. The excision required about 30 seconds for each brain and 30 to 60 seconds for ganglia. The extracts of 2 or 3 brains, or of 6 to 12 ganglia, were boiled for one minute, cooled, and stored at − 20° C. (Welsh, 1943). The isolated ventricle of the quahog, *Venus mercenaria,* was used in the assay of cholinergic substances, according to the method of Welsh (1943). About one-third of the assays were performed on ventricles which had been exposed for 15 minutes to 10^{-6} *M* LSD-25 (lysergic acid diethyl amide, Sandoz Products Ltd.). As Welsh (1954) has shown, this drug excites the molluscan heart maximally and thereby provides an excellent preparation for the assay of inhibitors of the ventricular beat. LSD treatment also masks the action of any excitors which might be present in the tissue extract.

d. *Choline acetylase.* In these determinations brain homogenates were incubated in a solution containing the salts, substrates, and cofactors favoring the acetylation of choline. The incubation media contained eserine to inhibit ChE, which would otherwise hydrolyze the desired product. The presence of fluoride is also necessary to protect the added ATP by inhibiting ATP-ases.

For each assay six animals at the same stage of development were anesthetized with carbon dioxide. The brains were excised and ground in a small, conical,

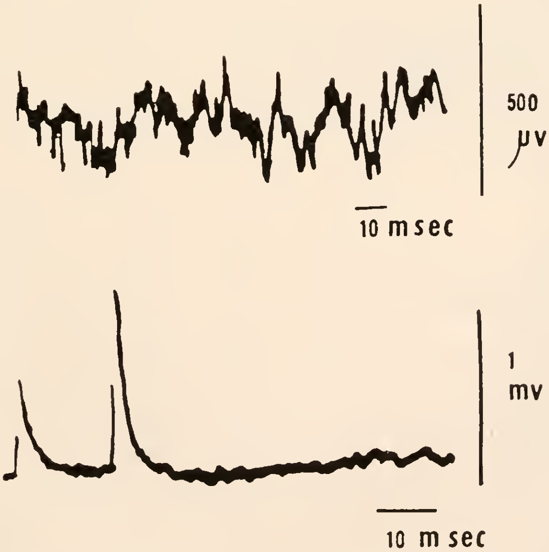

FIGURE 1. The electrical activity in the brain of a developing adult. The upper record was made with a fine stainless steel needle as the active electrode inserted into the brain and shows the summed potentials from many active neurons. The electrode for the lower record was an indium-filled micro-pipette with a tip diameter of approximately three microns. The action potentials of two individual neurons are shown.

glass homogenizer. The homogenate was diluted with 0.8 ml. of a solution containing the following reagents in the final concentrations specified: 18.7 mM choline chloride, 18.7 mM sodium acetate, 90 mM cysteine hydrochloride, 36.3 mM KCl, 0.9 mM $MgCl_2$, 1.9 mM Na_2HPO_4, 31.3 mM NaF, and 0.5 mM eserine sulfate; the pH was initially brought to 7.0 by the addition of HCl (Nachmansohn and Wilson, 1951; Gardiner and Whittaker, 1954). After equilibration of the reaction mixture in a water bath at 25° C., 0.1 ml. of 60 mM $ATP.Na_2$ (Pabst Labora-

TABLE I

The electrical activity of the brain during diapause and adult development

Stage of the animals	No. of observations	No. with active brains	No. with inactive brains
Platysamia cecropia			
Larvae—Days after beginning of cocoon construction			
0 days	4	4	0
2 days	5	5	0
5 days	5	5	0
7 days	5	5	0
9 days	3	0	3
10 days (Pupal moult)	5	0	5
Unchilled diapausing pupae	17	0	17
Diapausing pupae after storage at 6° C. for			
8 weeks	5	0	5
14 weeks	2	0	2
54 weeks	3	0	3
Pupae chilled for 8 weeks at 6° C. and then placed at 25° C. for 13 days	3	3	0
Pupae chilled for 10 weeks at 6° C. and then placed at 25° C. for 7 days	3	3	0
Pupae chilled for 13 weeks at 6° C. and then placed at 25° C. for			
0 days	3	0	3
2 days	2	0	2
4 days	2	0	2
6 days	3	2	1
8 days	3	3	0
During adult development			
0 day	2	2	0
1st day	2	2	0
2nd day	5	5	0
7th day	3	3	0
14th day	6	6	0
19th day	4	4	0
adult moth	4	4	0
Telea polyphemus			
Diapausing pupae	3	0	3
Developing adults	5	5	0
Samia walkeri			
Diapausing pupae	2	0	2
Developing adults	4	0	4

tories) and 0.1 ml. of a solution containing 360 Lipmann units of Coenzyme A (Pabst Laboratories) were added. After one hour incubation, dilute HCl was added to lower the pH to 4, and the mixture was boiled. The sample was then stored at − 20° C. and ultimately thawed and assayed on the *Venus* ventricle. The assay was controlled by identical determinations carried out on a mixture containing homogenate and all reagents except ATP.

Results

1. *The electrical activity of the central nervous system*

Figure 1 illustrates the electrical activity of the brain of the post-diapausing insect as recorded by two different techniques. The upper record was made with a stainless steel electrode, and shows the complex pattern of electrical activity generated by the "spontaneous" activity of many brain neurons. The second record, made with a microelectrode, shows the spontaneous activity of only two neurons. These records are typical of the electrical activity regularly detected in the brains of caterpillers and in the brains of animals after the end of diapause (Table I).

It is of special importance, as indicated in Table I, that no trace of activity could be recorded from the brains of diapausing pupae or of chilled pupae. This fact was confirmed, not only on Cecropia, but also on *Telea polyphemus* and *Samia walkeri*. Moreover, electrical stimulation of the ventral nerve cord or the subesophageal connective failed to evoke a response in the brain. Indeed, it was impossible to record any electrical response of the brain even when the brain was stimulated directly. Clearly most, and perhaps all, of the neurons of the diapausing brain are electrically inexcitable—the only uncertainty in this conclusion being the possibility that potentials from the smallest fibers of the insect brain were below the noise level of the amplification system. In summary, we see in the electrical measurements a profound difference between the brains of diapausing and post-diapausing pupae. This difference, in my experience, was clear cut: either the brain was electrically active or it was electrically silent.

As recorded in Table I, the electrical activity characteristic of the larval brain is already absent by the day before the pupal molt. The electrical silence then persists in pupae stored for as long as six months at 25° C. Indeed, the brains were still electrically inactive in pupae stored for a year at 6° C. Apparently, even prolonged chilling does not restore electrical activity to the brain. It will be noted in Table I that when pupae chilled for a long time were placed at 25° C. the electrical activity of the brain reappeared after only a few days. For example, in pupae stored at 6° C. for 13 weeks, the activity first appeared on the sixth day after transfer to 25° C. This corresponds to three days before the first visible signs of the onset of adult development.

It is of considerable interest that the electrical inactivity of the diapausing pupa is confined to the brain. Detailed electrical studies showed that all of the ganglia of the ventral nerve cord, from the subesophageal ganglion to the eighth abdominal ganglion, retained spontaneous activity throughout diapause, and that the ventral nerve cord always responded to electrical stimulation.

2. *Cholinesterase in the central nervous system*

The endocrinologically inactive brain of the diapausing pupa has been found to be electrically silent. Electrical activity disappears from the brain just before pupation. And the resumption of development at the end of diapause is prefaced by the reappearance of the brain's electrical activity. The electrical changes lead one to anticipate corresponding changes in the chemical mechanism underlying axonal conduction and synaptic transmission. For this reason the behavior of ChE was studied by the techniques described in the section on Methods.

As shown in Figure 2, the brains of fourth instar larvae contain a significant titer of ChE. ChE remains constant in the larval brain during the molt to the fifth instar and then slowly increases to the high titer characteristic of the brains of spinning caterpillars. After completion of the cocoon, the high titer of ChE persists in the brain for six days. Then, in the two days remaining before the pupal molt, the brain's ChE falls precipitously and becomes undetectable two days later. It is interesting that the fall in ChE is synchronous with the loss of the brain's electrical activity.

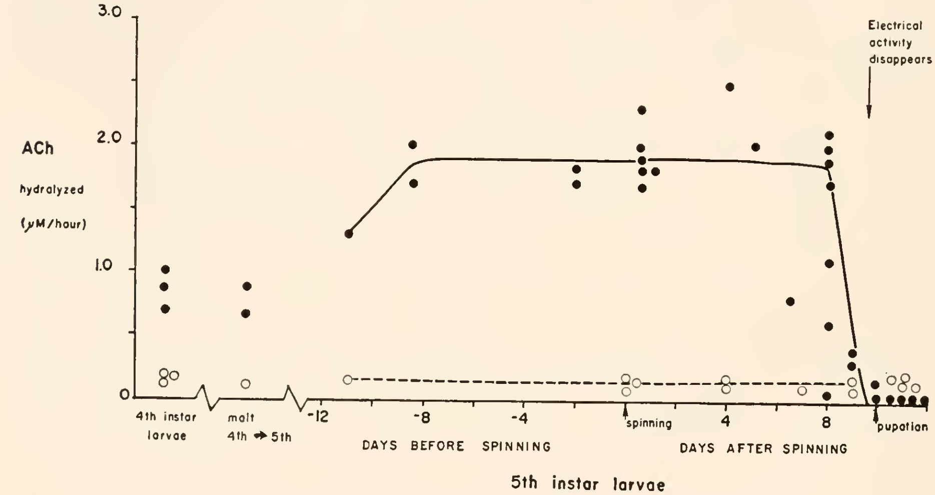

FIGURE 2. The cholinesterase activity of larval brains and ganglia. The ordinate shows the amount of ACh hydrolyzed per brain (or ganglion) in one hour. ●, brains; ○, ganglia.

Figure 2 also shows that the ganglia of the ventral nerve cord maintain a uniform titer of ChE throughout the final larval instar and the pupal stage that follows. In short, the virtual absence of ChE is characteristic of only the brains of diapausing pupae. Of eight brains from fresh pupae subjected to individual analysis, only one showed any detectable ChE. Even this exceptional brain contained only a trace, corresponding to the hydrolysis of 0.07 μM of ACh per hour. Similarly, ChE was undetectable in individual determinations on three diapausing brains of *Telea* and on five brains from diapausing *Samia*.

Prolonged exposure of diapausing Cecropia to low temperature is without detectable effects in restoring the brain's ChE. Fourteen determinations on a total of 44 brains from pupae chilled at 6° C. showed that cholinesterase remained undetectable, even though two of the determinations were on the brains of pupae chilled for as long as 30 weeks. Consequently, it is clear that the diapausing brain, even after prolonged chilling, is characterized by a ChE titer undetectable by the present methods. In the preceding section these brains were likewise found to be electrically silent.

In the brains of previously chilled pupae ChE becomes detectable after a few days at 25° C. For example, Figure 3 illustrates results obtained on a series of pupae previously chilled for 13 weeks at 6° C. When the animals were placed at 25° C. and their brains analyzed at successive intervals, ChE remained undetectable for the first four days. Then, on the fifth day, brain ChE became barely detectable. Thereafter the activity increased rapidly until the onset of adult development, and then more slowly. Apparently, a slight but definite decline in ChE occurs prior to the emergence of the adult moth. Figure 3 likewise makes clear that the return of detectable titers of brain ChE correlates in time with the appearance of electrical activity.

Similarly, a total of six individual determinations on the brains of developing *Telea* and eight determinations on the brains of developing *Samia* showed that the post-diapausing brains of these species contained finite titers of ChE.

It will be recalled that the ventral nerve cord retains electrical activity throughout diapause. The ganglia from diapausing or chilled pupae also retained ChE, hydrolyzing an average of 0.13 ± 0.05 μM of ACh per ganglion per hour. As recorded in Figure 3, the enzymatic activity of the ganglia remains uniform throughout the entire course of adult development.

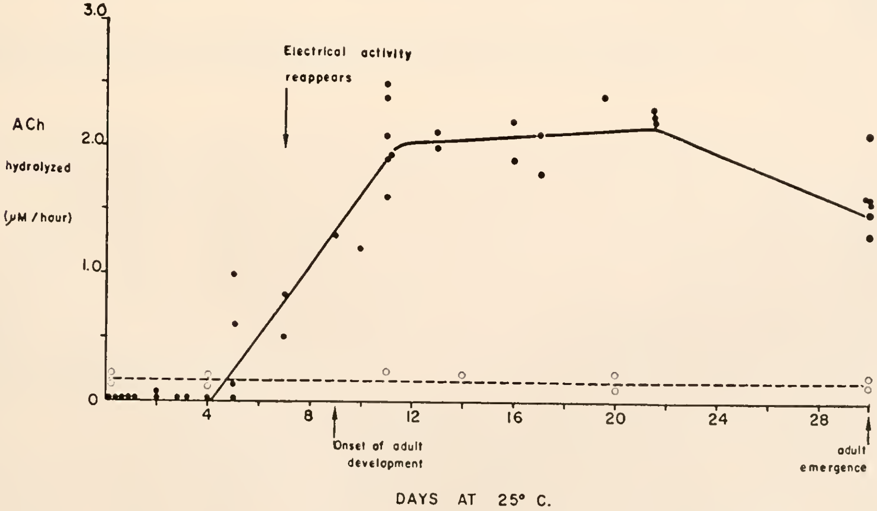

FIGURE 3. Cholinesterase activity in the brains and ganglia of pupae chilled for 13 weeks and then brought to 25° C. The ordinate shows the amount of ACh hydrolyzed per brain (or per ganglion) in one hour. ●, brains; ○, ganglia.

3. *The absence of a cholinesterase inhibitor*

In order to exclude the possibility that some inhibitor in the diapausing brain blocks the action of ChE, homogenates containing ChE were prepared, each containing the brains of two non-diapausing animals. The brei of a diapausing brain was added to half of each homogenate, and the ChE activities of both the remaining half of the original homogenate and the homogenate plus diapausing brain were measured. There was no evidence of inhibition in three experiments in which brains from newly diapausing pupae were added, or in three experiments in which chilled diapausing brains were added.

4. *Cholinesterase and electrical activity in the brain of a non-diapausing insect*

It seemed possible that the disappearance of electrical activity and of cholinesterase in the brain of the diapausing pupae might merely reflect a prolongation, by the intervention of diapause, of a sequence of events which normally occurs during the metamorphosis of non-diapausing species. To examine this possibility, the brain of *Galleria,* a non-diapausing lepidopteran, was studied. At 25° C. *Galleria* requires ten days from the pupal molt to the emergence of the adult moth; the true pupal stage is only the first 30 hours of this period (Schneiderman, 1952). Accordingly, electrical observations were made on the brains of six *Galleria* pupae selected during the first eight hours after the pupal molt. All of the brains were electrically active.

In further experiments, two *Galleria* were collected on each of the first six days after the pupal molt. The brains were excised and individually assayed for ChE. The titer of ChE activity corresponded to 0.9 ± 0.2 μM of ACh hydrolyzed per brain per hour; the activity showed no correlation with the stage of adult development.

5. *The effect of prothoracic gland hormone on the resynthesis of cholinesterase by the brain*

The results reported so far show that ChE is first detected in the pupal brain a few days before the onset of adult development; that is, at the beginning of the period when the brain's neurosecretory cells are probably maximally active. Consequently, it was important to decide whether the reappearance of brain ChE was a cause or a result of the recovery of the brain's endocrine action. The method, in brief, was to determine whether ChE appeared in previously chilled brains cultured *in vivo* in the absence of prothoracic gland hormone. To this end, a series of diapausing pupal abdomens were prepared from Cecropia pupae by the method of Williams (1947). Two weeks later, two or three pupal Cecropia brains were implanted into each abdomen and the preparation stored at 25° C. After two or four weeks the brain implants were removed and assayed for ChE.

The results, summarized in Table II, show that the synthesis of ChE took place in the previously chilled brains during the period of incubation in isolated abdomens. But unchilled brains under similar conditions were unable to synthesize ChE. Apparently, the synthesis of ChE is dependent on prior exposure to low temperature but is not dependent on the presence of the prothoracic gland hormone.

TABLE II

Cholinesterase in brains after incubation in isolated abdomens

Temperature treatment of the brain donors	Length of incubation in isolated abdomens	ACh hydrolyzed (μM/brain/hour)
Stored continuously at 25° C.	2 weeks	0
		0
		0
		0
		0
		0.2
Stored at 6° C. for 13 weeks	2 weeks	0.6
		0.7
		0.9
		1.0
Stored at 6° C. for 18 weeks	2 weeks	1.6
		1.8
Stored at 6° C. for 18 weeks	4 weeks	1.9
		2.0
		2.0

This conclusion was reinforced in a second series of experiments testing whether the brain hormone or the prothoracic gland hormone could promote the appearance of ChE in unchilled pupal brains. Brains from unchilled pupae were exposed to these hormones by implanting them into the tip of the abdomen in previously chilled pupae. The latter were then placed at 25° C. and allowed to begin adult development. On the second day of adult development the implanted brains were recovered and assayed for ChE. Five experiments performed on individual brains showed that the unchilled brains did not acquire detectable ChE when exposed in this manner to both the brain and prothoracic gland hormones.

6. *The cholinergic substance in brains and ganglia during diapause*

The remarkable variation in the ChE activity of the brain encouraged a study of the changes occurring in the corresponding cholinergic substrate during diapause and development. Results based on the *Venus* heart assay are summarized in Figure 4. Brain extracts from newly pupated animals showed only a trace of cholinergic activity. In marked contrast to the behavior of ChE, the brain's cholinergic activity undergoes a definite increase during diapause, the rate of increase being slow at 25° C. and rapid at 6° or 15° C. As mentioned in the section on Methods, the fact that about one-third of the *Venus* hearts had been treated with LSD-25 gives assurance that the changes are due to an increase in cholinergic substance rather than to a decrease in adrenergic substance.

As shown in Figure 5, the changes in the cholinergic substance during diapause are peculiar to the brain. The ganglia of the ventral nerve cord maintain a uniform titer of cholinergic substance during diapause, irrespective of the temperature of storage or of the duration of diapause.

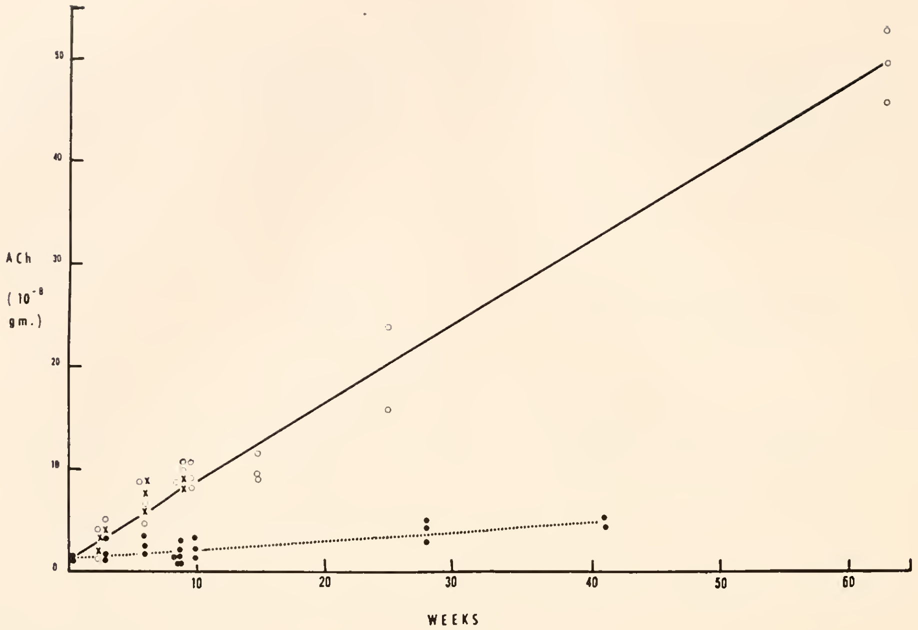

FIGURE 4. The titer of cholinergic substance in the brain as affected by the duration of diapause and the temperature or storage. The titer is expressed as the amount of ACh.Cl possessing identical action on the *Venus* ventricle. ○, stored at 6° C.; ×, stored at 15° C.; ●, stored at 25° C.

7. *The cholinergic substance during the reactivation of the brain*

In the preceding section the diapausing brain was found to acquire a considerable titer of the cholinergic substance during chilling. Figure 6 summarizes the history of the cholinergic material subsequent to the transfer of the chilled pupae from 6° to 25° C. Cholinergic material persists at high concentrations during the

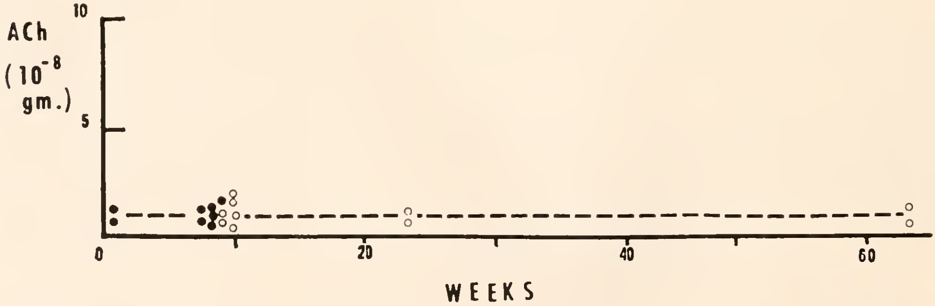

FIGURE 5. The titer of cholinergic substance in pupal ganglia as affected by the duration of diapause and the temperature of storage. Ordinate as in Figure 3. ○, stored at 6° C.; ●, stored at 25° C.

first few days at 25° C. and then undergoes a precipitous decrease. This sudden drop in cholinergic substance coincides in time with the reappearance in the brain of detectable ChE. The cholinergic substance then remains at the low and apparently constant level until just prior to adult emergence, and then increases five-fold to the high level characteristic of the adult brain.

Figure 6 also records corresponding measurements of the cholinergic substance in the ganglia of the same animals which served as donors of the brains. Here again, the changes are peculiar to the brain and are not encountered in the ganglia.

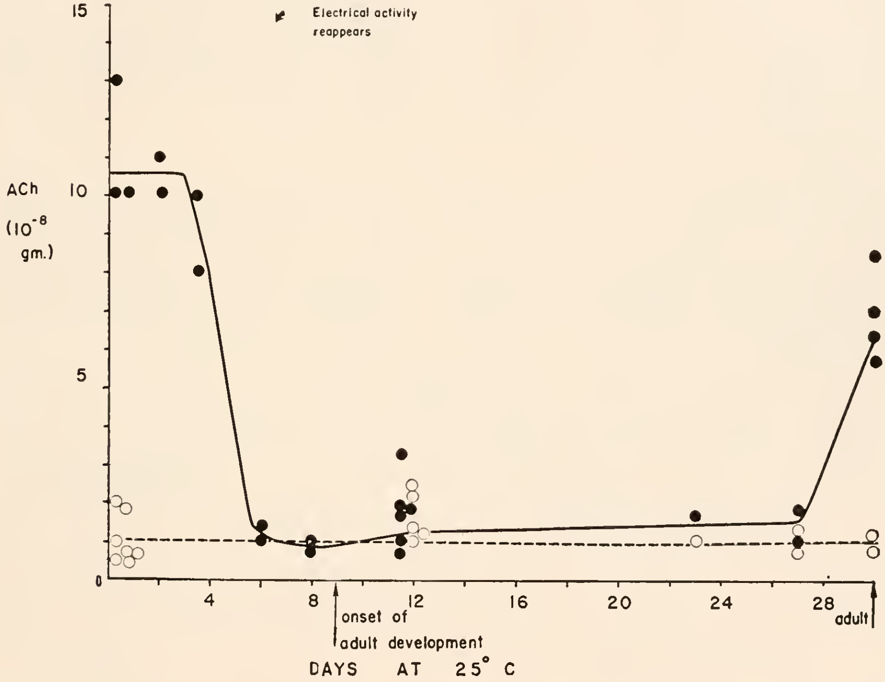

Figure 6. The titer of cholinergic substance in the brains and ganglia of pupae chilled for 13 weeks and then brought to 25° C. Ordinate as in Figure 3. ●, brain; ○, ganglia.

8. *The nature of the cholinergic substance*

Though the nature of the cholinergic substance has not been explored in detail, two observations show that the substance from Cecropia has certain resemblances to ACh. Cecropia brain extracts failed to depress the beat of *Venus* ventricles which had been previously treated with 0.01 per cent "Mytolon" [2 : 5-bis(3′diethylaminopropylamino)-benzoquinone bis-benzyl chloride, Sterling-Winthrop], a drug which has been shown by Welsh and Taub (1953) to block the action of ACh and related compounds by combining with the specific protein receptor in the *Venus* ventricle. Furthermore, the compound from Cecropia shares acetylcholine's properties of being promptly destroyed by boiling at alkaline pH's, but resistant to boiling at acid pH's.

9. *Adrenergic substances in the Cecropia brain*

In view of the marked changes in the brain's cholinergic substance during diapause and development, there remained the possibility that molecules with an adrenergic action might also correlate with this change. Fortunately, as discussed above, "Mytolon" blocks the action of cholinergic substances on the *Venus* ventricle so that the heart after this treatment becomes suitable for the assay of adrenergic agents. A total of 18 pupal brains was prepared in six equal extracts and tested by this technique. Negative results were obtained in all cases, irrespective of the stage of the donors. The brain appears to contain no detectable adrenergic agent. However, the term "detectable" signifies very different concentrations of the various adrenergic agents: as high as 5×10^{-5} *M* for adrenaline, as low as 10^{-10} *M* for 5-hydroxytryptamine (Welsh, 1953).

10. *Choline acetylase in diapausing and developing brains*

Since the synthesis of ACh and perhaps of other cholinergic substances is catalyzed by choline acetylase, the changes observed in the brain's ACh might be secondary to changes in the choline acetylase. This possibility was explored by assaying the choline acetylase concentration according to the technique described under Methods.

The results, summarized in Table III, show that choline acetylase is present in

TABLE III

Choline acetylase activity in brains of animals at various stages

Stage of the animals	ACh synthesized ($\times 10^{-8}$ gm./brain/hour)
Unchilled diapausing pupae	0.6
	0.8
	1.2
	1.7
	3.1
Pupae after 9 weeks of storage at 6° C.	0.8
	1.5
	1.6
	1.7
	1.7
	2.1
Pupae chilled for 13 weeks at 6° C. and then placed at 25° C. for 8 days	1.3
	2.5
Zero day of adult development	1.0
	3.0
Second day of adult development	2.1
	6.2
	7.5
Seventeenth day of adult development	3.5
	6.2

the brain throughout pupal diapause and adult development. Furthermore, its titer in the pupal brain shows no correlation with the previous temperature history of the animal, or with the reappearance of ChE, or with the onset of electrical excitability. However, as Table III reveals, a prominent increase in the choline acetylase of the brain takes place after the onset of adult development.

Discussion

1. *The physiology of the brain during metamorphosis*

In Figure 7 the results of the present study are summarized and compared with the recurrent periods of endocrine activity of the brain as estimated from the studies of Williams (1946b, 1952, unpublished data) and Schneiderman and Williams (1953). The figure makes clear the parallel between the activity of the brain's neurosecretory cells and the physiology of the brain as a whole. It will be observed that the brain of the fifth instar caterpillar is endocrinologically active, electrically active, and contains a high titer of ChE. All of these parameters decline rapidly in the brain of the pre-pupa, so that by the time of the pupal molt the brain is endocrinologically inactive (Williams, 1952), electrically silent, and devoid of detectable ChE.

The electrical and enzymatic defects in the brain persist throughout the months of pupal diapause, and during this same period the brain is thought to be endocrinologically inactive. However, Williams (unpublished data) has recently shown that during prolonged chilling at 6° C. the neurosecretory cells apparently begin to release small quantities of brain hormone, though only after a year or longer at the low temperature does this "leakage" of hormone become sufficient to trigger adult development. In the present study the sole change found in the brain of the diapausing pupa was the progressive accumulation of cholinergic substance. This accumulation, as we have seen, is slow at room temperatures and rapid at low temperatures. The significance of this result will be discussed below.

As Figure 7 also shows, when a chilled pupa is transferred to 25° C. the brain is promptly reactivated. The first change detected is the appearance of measurable titers of brain ChE. Simultaneously, the cholinergic substance in the brain falls

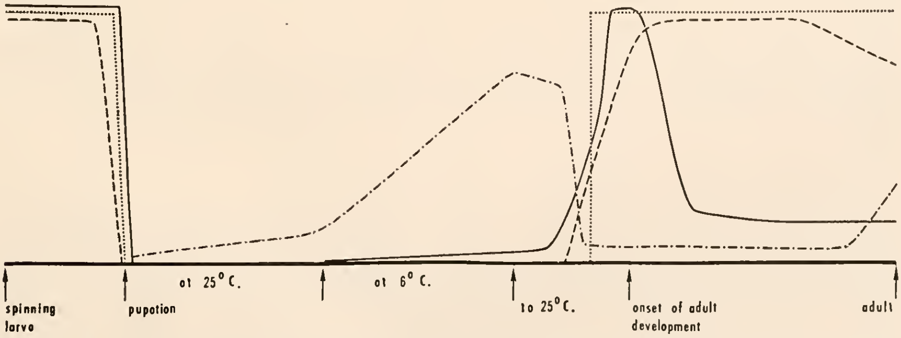

FIGURE 7. A diagrammatic summary of the physiology of the brain and the release of brain hormone during metamorphosis. The time scale during diapause is, of course, greatly condensed. For explanation see text. —, release of brain hormone; · · ·, electrical activity of the brain; — —, ChE in the brain; · —, titer of cholinergic substance in the brain.

precipitously from the high titer accumulated during chilling to the low titer characteristic of adult development. Immediately after the reappearance of ChE the brain regains electrical activity.

It is impossible to establish with the same precision the time when the neurosecretory cells begin to secrete the brain hormone. In the absence of a direct assay of the brain hormone it is necessary to infer the time of its release from indirect evidence. Schneiderman and Williams (1953) have estimated the time of release of prothoracic gland hormone. Their estimate is based on measurements of the oxygen consumption of previously chilled pupae during the interval between transfer to 25° C. and the onset of adult development. The results show that the oxygen consumption of such pupae begins a progressive increase about seven days before the onset of adult development. Schneiderman and Williams suggest that the increase in oxygen consumption results from the action of prothoracic gland hormone. Since the brain hormone is prerequisite for the function of the prothoracic glands, we are led to infer that the release of brain hormone begins more than seven days before the onset of adult development at 25° C. But in the present study the first changes in the physiology of the brain begin only five days before the onset of adult development. However, it is probably not prudent to emphasize this minor discrepancy between the data of Schneiderman and Williams and the present results, because the previous temperature histories of the pupae studied were quite different.

As Williams (1946b) demonstrated, the brain's neurosecretory cells are maximally active only until the first days of adult development. Activity then declines. Originally it was believed that at this time the neurosecretory cells lost all activity, but in recent extensive studies Williams (personal communication) found that in the adult brain the neurosecretory cells may maintain a low rate of hormone release. By contrast, the brain's ChE progressively increases from the time of reappearance until a maximum is reached on the second day of adult development. The titer is maintained until shortly before the emergence of the adult moth, when a decrease in brain ChE takes place. On the other hand, the cholinergic substance in the brain remains at a uniform low titer throughout adult development and finally increases five-fold shortly before the emergence of the adult moth. These changes, as we have seen, are without effect on the brain's electrical activity, which continues unaltered during adult development.

It is worth recalling that all of the changes shown in Figure 7 are peculiar to the brain. Throughout metamorphosis the ganglia of the ventral nerve cord retain electrical activity, ChE, and a uniform titer of cholinergic substance.

2. *Cholinesterase and the electrical activity of the brain*

The results show that the titer of ChE during metamorphosis is paralleled by changes in the electrical activity of the brain. At pupation, ChE becomes undetectable and the brain becomes electrically silent. Months later, the return of detectable ChE is followed by the return of electrical activity to the brain. This striking correlation is interesting in view of the considerable evidence, summarized by Nachmansohn (1953–54), that ChE is essential for conduction in axons. In any event it seems probable that the disappearance of ChE can, in itself, account for the loss of electrical activity of the Cecropia brain.

It is possible, of course, that the diapausing brain loses enzymes in addition to ChE. However, the results show that choline acetylase, an enzyme of the same metabolic cycle, persists in the brain throughout diapause; indeed, the titer of choline acetylase appears to remain unchanged as electrical activity returns to the brain. At present there is no reason to suppose that diapause is accompanied by profound changes in any of the brain's enzymes except ChE.

3. *Electrical activity and brain hormone release*

Since no direct assay is known for the brain hormone, it is difficult to correlate precisely the electrical activity and the release of brain hormone. At the onset of diapause the endocrine competence and the electrical activity of the brain are lost simultaneously. Here the electrical inactivity of the brain can account for the failure of neurosecretion and the consequent onset of diapause. At the end of diapause the situation is less conclusive. It seems clear from the available facts that the rapid release of brain hormone before the onset of adult development occurs at about the time when the brain regains electrical activity.

These inferences are supported by the investigations of the causes of the reappearance of ChE in the brain. It will be recalled that previously chilled brains were able to synthesize ChE, even when cultured in the absence of the prothoracic gland hormone. Unchilled brains under the same circumstances were unable to synthesize ChE even when exposed to the brain and prothoracic gland hormones. Williams (personal communication) has already shown that unchilled pupal brains fail to regain endocrine competence when exposed to brain and prothoracic gland hormones. Apparently, the factors which promote the return of ChE to the brain are precisely the same as the factors which elicit the return of endocrine activity.

4. *The changes in the brain during diapause*

The results show that the reappearance of detectable ChE and of endocrine activity is hastened by a few weeks of exposure to low temperatures. The low temperatures favor the rapid accumulation of a cholinergic substance in the pupal brain. When a high titer of cholinergic substance is present in the brain, the neurosecretory cells are promptly reactivated when returned to 25° C. The analysis so far suggests that the reactivation of the neurosecretory cells depends on the synthesis of ChE and the consequent establishment of electrical activity in the brain as a whole. It therefore seems possible that the high titer of cholinergic substance triggers the prompt synthesis of ChE; that is, the synthesis of ChE may be an adaptive response to high substrate concentrations. As Featherstone *et al.* (1955) have shown, the adaptive synthesis of ChE can be elicited in chick intestine cultivated *in vitro.*

The evidence suggests that the pupal diapause of Cecropia results from the failure of the brain as a whole. The diapausing brain is electrically silent, apparently because of the absence of ChE. Evidently, it is essential for the pupa to inactivate the entire brain to insure the persistent inactivity of the neurosecretory cells. ChE is slowly synthesized by the brain after prolonged storage at room temperatures, but promptly following storage at low temperatures. Low temperatures favor the rapid accumulation of a cholinergic material in the brain which suggests that the reappearance of ChE is an adaptive response to high substrate concentrations. When ChE reappears, the brain becomes electrically active, the neurosecretory cells release brain hormone, and the long season of diapause is ended.

Professor Carroll M. Williams, of Harvard University, aided this investigation by sharing his unpublished observations, by many helpful discussions, and by a critical reading of the typescript of this paper. It is a pleasure to acknowledge my gratitude. I am also indebted to Professor J. H. Welsh, of Harvard University, for advice on ACh assay techniques and for providing several of the drugs used in the assays.

SUMMARY

1. The 26 neurosecretory cells in the brain of the Cecropia silkworm have been shown by Williams to undergo systematic changes in endocrine activity during metamorphosis. At the pupal molt the neurosecretory cells lose endocrine activity. Consequently, growth is checked and the animal enters pupal diapause. Endocrine activity is restored following prolonged exposure to low temperatures. When the brain hormone is released, diapause ends and the development of the adult moth begins.

2. The electrical events in the brain as a whole have been studied in relation to these changes in endocrine activity. The larval brain shows abundant "spontaneous" electrical activity. However, on the day before the pupal molt the brain loses spontaneous activity and is no longer excited by direct electrical stimulation. The electrical silence of the brain persists throughout the months of diapause. Electrical activity reappears in the pupal brain a few days before the end of diapause.

3. Cholinesterase (ChE) is present in high titer in the larval brain. However, two days before pupation the brain ChE begins a rapid decline, so at pupation ChE is undetectable in the brain. ChE does not reappear in the pupal brain until shortly before the recovery of electrical and endocrine activities. There is no evidence that an inhibitor of ChE is present in the diapausing brain. Evidently, ChE is absent or in low titer and is synthesized just before the onset of adult development.

4. During diapause the cholinergic action of brain extracts steadily increases. The increase is slow in the brains of pupae stored at 25° C. and rapid in the brains of pupae stored at 15° or 6° C. Therefore the rate of accumulation of the cholinergic substance parallels the effectiveness of the temperature treatment in restoring the brain to endocrine activity.

5. When ChE reappears in the brain, just before the onset of adult development, the titer of cholinergic substance in the brain falls to a low level. The low level is maintained until shortly before adult emergence, when the titer of cholinergic substance increases five-fold.

6. Choline acetylase activity remains uniform in brain homogenates from unchilled and chilled pupae.

7. The changes in electrical activity, ChE, and cholinergic substance during metamorphosis are peculiar to the brain. The ganglia of the ventral nerve cord retain normal function throughout diapause.

8. ChE is synthesized in chilled pupal brains which are cultured at 25° C. in the absence of prothoracic glands. Under the same circumstances unchilled brains are unable to synthesize ChE. Moreover, exposure to the brain and the prothoracic gland hormones fails to promote the synthesis of ChE in unchilled pupal brains.

9. ChE and electrical activity remain constant during the metamorphosis of *Galleria,* a non-diapausing insect. Two additional diapausing species, *Telea polyphemus* and *Samia walkeri* showed variations in brain ChE and electrical activity identical to those described in Cecropia.

10. The evidence suggests that the pupal diapause of Cecropia results from the failure of the brain as a whole. The diapausing brain is electrically silent, apparently because of the absence of ChE. During diapause there is an accumulation of a cholinergic substance in the brain—an observation which suggests that the reappearance of ChE is elicited by high substrate concentrations. When ChE reappears, the brain becomes electrically active, the neurosecretory cells release brain hormone, and diapause is ended.

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ADENOSINETRIPHOSPHATASE OF MYTILUS SPERMATOZOA. I. EFFECTS OF pH, CALCIUM AND MAGNESIUM, AND CONCENTRATION OF ENZYME AND SUBSTRATE

LEONARD NELSON

Department of Physiology, University of Nebraska, Lincoln, Nebr., and Marine Biological Laboratory, Woods Hole, Mass.[1]

The flagellum is the motile organelle of the sperm cell. Motility is apparently independent of the immediate control of the head. Inactivation of the nucleus, as by the action of dyes on frog sperm, causes no damage to the extranuclear parts, and eggs inseminated with the treated sperm may go on to develop haploid embryos (Briggs, 1952); waving movements of sperm tails detached from heads have also been observed (Rothschild, 1953). It is, therefore, not surprising that an enzyme capable of splitting ATP has been found concentrated in the flagella of the sperm of certain marine invertebrates and of the bull (Nelson, 1951, 1954a).

Investigations into the nature of the enzyme associated with movement have included a variety of studies in other biological systems. These range from the effects of ATP on contraction of isolated bacterial flagella (DeRobertis and Franchi, 1952), on pseudopod formation and protoplasmic streaming in ameba (Goldacre and Lorch, 1950), and on rate of ciliary activity of frog pharyngeal mucosa (Vorhaus and Deyrup, 1953) to the effects of the cation composition and pH of the incubation medium on ATP dephosphorylation by vertebrate and insect muscle ATP-ase. A calcium-activated ATP-ase has been derived from the myosin component of muscle fibrils (Banga and Szent-Györgyi, 1943), as distinguished from a soluble magnesium-activated ATP-ase readily extractable from both vertebrate (Kielley and Meyerhof, 1948) and insect muscle (Gilmour and Calaby, 1952; Maruyama, 1954). Combination with actin has been found to reverse magnesium-inhibition, as well as to alter the pH optimum, of myosin ATP-ase (Sarkar et al., 1951).

The function of ATP in spermatozoa has been examined by a number of investigators (*cf.* reviews by Mann, 1949, 1954), all of whom conclude that ATP plays an important role in sperm motility. Depletion of the ATP content of semen coincides with impairment and cessation of motility of ejaculated bull spermatozoa (Mann, 1945). The sperm tail enzyme, tentatively referred to as adenylpyrophosphatase, preferentially hydrolyzed ATP, and was relatively inactive toward yeast adenylate, fructose diphosphate and beta-glycerophosphate (Nelson, 1954a). Apyrase may be extracted from bull sperm tails with glass-distilled water or 0.6 *M* KCl. While the KCl extracts nearly all of the enzyme, a high degree of activity remains with the residue of the water-extracted material. On the other hand, Mytilus sperm tails resist extraction by either of these methods. However, dilution of KCl or sea water suspensions with large quantities of ice-cold distilled water leads to agglutination and precipitation of the Mytilus sperm tails (Nelson, 1954b).

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[1] With the technical assistance of Abolghassem Amin and Anne Ruth Nelson.

In order further to characterize the nature of this enzyme, studies have been conducted on the influence of pH, calcium and magnesium, and enzyme and substrate concentration on the rate of ATP dephosphorylation by Mytilus sperm tail preparations.[2] The results of these experiments are presented in this paper.

MATERIALS AND METHODS

The testes of three or four bay mussels, *Mytilus edulis,* were excised and minced in a small volume of filtered sea water (FSW), and the sperm was separated from the mince by washing gently through four layers of cheese cloth. The sperm suspension was concentrated at a relative centrifugal force of 2000 × gravity for five minutes. The supernate was decanted and discarded and the sperm resuspended in 10 volumes of FSW; the washing is repeated several more times in either FSW or *M*/2 KCl. The spermatozoa may be "decapitated" by either of two methods: a) the sperm are placed in a chilled Pyrex mortar in the deep freeze until the suspension acquires a slushy consistency, at which time the suspension is ground with a Pyrex pestle for two minutes; or b) the suspension is diluted with FSW and run through a Logeman stainless steel hand homogenizer. Spinning the homogenate at 1000 × g for ten minutes separates the heads from the tails, which remain in the supernate. The residue may be re-suspended two more times in FSW or isotonic KCl and the centrifugation repeated, thereby increasing the recovery of the tails. The supernates are then pooled and the tails collected by centrifuging at 7000 × g for ten minutes. The supernate is discarded and the tails are then suspended in *M*/2 KCl. (All these operations are carried out in the cold room, or with equipment which has been chilled in the refrigerator, and ice-cold solutions employed.)

Phosphate liberation is measured according to Moog and Steinbach (1945) using a modification of the colorimetric method of Taussky and Shorr (1953) in the Klett-Summerson colorimeter with the No. 66 filter. The reactants are mixed in 12-ml. Pyrex conical centrifuge tubes and equilibrated in the thermostat at 26–27° C. for at least 5 minutes prior to the initiation of the experiment by the addition of the ATP. Each tube contained 0.2 ml. sample, and, in final concentrations, 0.05 *M* KCl, 0.01 *M* veronal buffer, 8×10^{-4} *M* or 1.6×10^{-3} *M* ATP[3] (0.1 or 0.2 mg. 7′-phosphate) plus glass-distilled H_2O and $MgCl_2$ or $CaCl_2$ to make a total volume of 2.0 ml. After incubation for 10 minutes in the thermostat, the reaction was terminated by the addition of 2.0 ml. ice-cold 10% trichloroacetic acid, the precipitate removed by centrifugation, and the entire supernate analyzed for orthophosphate. Reagent blanks are run in duplicate tubes to which the trichloroacetic acid was added prior to the ATP. Controls for the experiments on the influence of divalent cations included all the components of the reaction mixture except the magnesium or calcium chlorides, and were also run simultaneously with the experimentals. In these determinations, the results are expressed as per cent of the control phosphate liberation. In the studies on the influence of pH, and the effect of enzyme and substrate concentration, the results are stated in terms of specific activity, micrograms of phosphorus liberated per milligram of nitrogen per minute (N determined by the semi-micro-Kjeldahl method of Ma and Zuazaga, 1942).

[2] This work was partially supported by grants from the University of Nebraska Research Council.

[3] Disodium ATP (Pabst), neutralized with NaOH.

Results

Effects of the divalent cations. The mode of preparation of the sperm affects the response of the tail enzyme to the divalent cations, calcium and magnesium. This is illustrated in Figure 1, where the enzyme activity of preparations washed 3 times in FSW and once in *M*/2 KCl is compared with that of sperm washed twice in FSW and 4 times in *M*/2 KCl. When the first washing procedure is followed, calcium inhibits in all concentrations, while the ATP-ase activity is maximal in 4×10^{-5} *M* $MgCl_2$. Not only is the optimum for magnesium shifted to a higher concentration, but calcium also now has a slightly activating effect on the enzyme when the latter method of washing is employed. Apparently a considerable amount of the divalent cations of the sea water remains with the residue when the sperm are washed only once in isotonic KCl after repeated washing in FSW. Since Woods

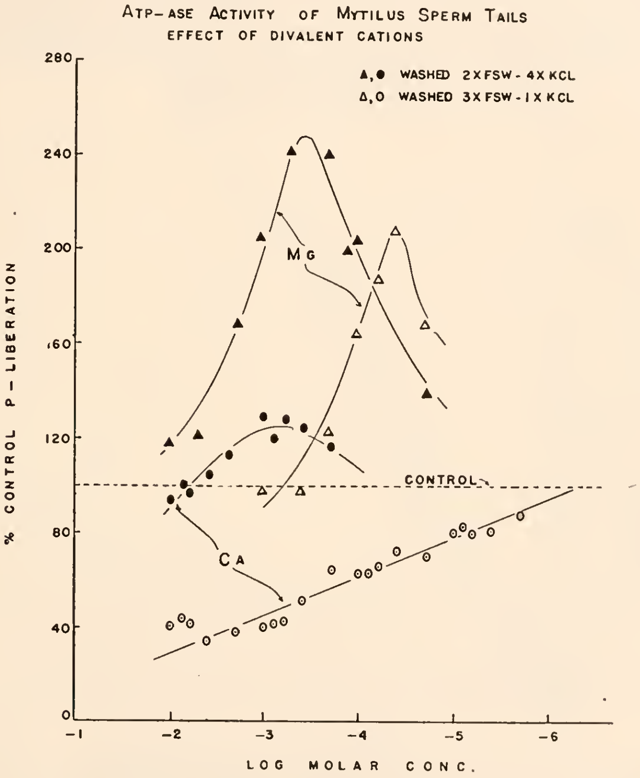

FIGURE 1. ATP-ase activity of Mytilus sperm tails. Effect of divalent cations. Sperm washed twice in filtered sea water (FSW) and 4 times in *M*/2 KCl ▲, ●. Sperm washed 3 times in FSW and once in *M*/2 KCl △, ⊙. Varying concentrations of $MgCl_2$ ▲, △. Varying concentrations of $CaCl_2$ ●, ⊙. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 8.4, 8×10^{-4} *M* ATP, $MgCl_2$ or $CaCl_2$, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 27° C. Ordinate—% control P-liberation, abscissa—log molar concentration divalent cation.

Hole sea water contains somewhat over 50 millimoles of magnesium, and about 9 millimoles of calcium per liter, this suggests that the residual cations following the single KCl wash not only contribute measurably to the ionic composition of the incubation medium, but also they exert mutually antagonistic effects on the enzyme action (an alternative mechanism is also indicated on the basis of the work of Tyler, 1953, that addition of amino acids, peptides, proteins and metal-chelating agents is beneficial to sperm by inducing complex formation with various toxic heavy metals commonly present in sea water). The present hypothesis may be tested on the 2 ×

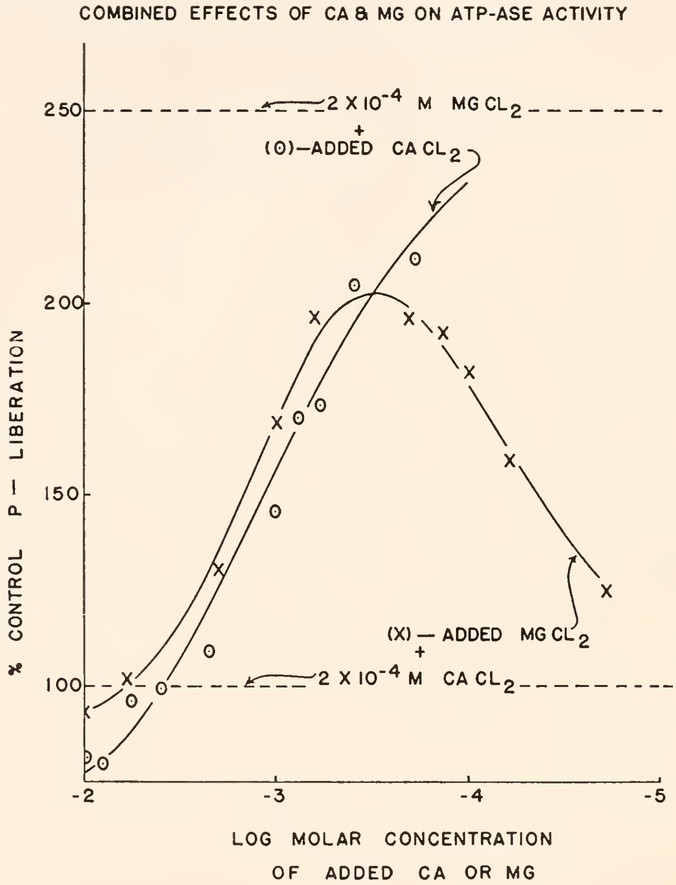

FIGURE 2. Combined effects of Ca and Mg on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in $M/2$ KCl. ⊙—$MgCl_2$, 2×10^{-4} M plus graded amounts of $CaCl_2$. ×—$CaCl_2$, 2×10^{-4} M plus graded amounts of $MgCl_2$. Tube contents: 0.05 M KCl, 0.01 M veronal buffer pH 8.4, 8×10^{-4} M ATP, $MgCl_2$ as indicated, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 27° C. Ordinate—% control (no divalent cation) P-liberation, abscissa—log molar concentration of Ca or Mg added in graded amounts to reaction mixture containing fixed concentration of other divalent cation.

FSW-4 × KCl wash preparations by adding varying amounts of one of the divalent cations to the reaction mixture containing a fixed concentration of the other. The results of such an experiment are summarized graphically in Figure 2. The fixed concentration of the cations was arbitrarily set at 2×10^{-4} M, since this was within the range of optimum activation by $MgCl_2$, and at this concentration the rate of ATP dephosphorylation in $CaCl_2$ was essentially the same as that of the control (no added divalent cation). All additions of Ca depressed the activation by 2×10^{-4} M $MgCl_2$; whereas the maximum antagonistic effect of Mg added to 2×10^{-4} M $CaCl_2$ obtained when the $MgCl_2$ reached about 3×10^{-4} M. Apparently the difference in the influence of the two divalent cations is not merely a matter of degree. Magnesium may potentiate the slightly stimulatory effect of calcium, while calcium in all concentrations antagonizes the magnesium effect.

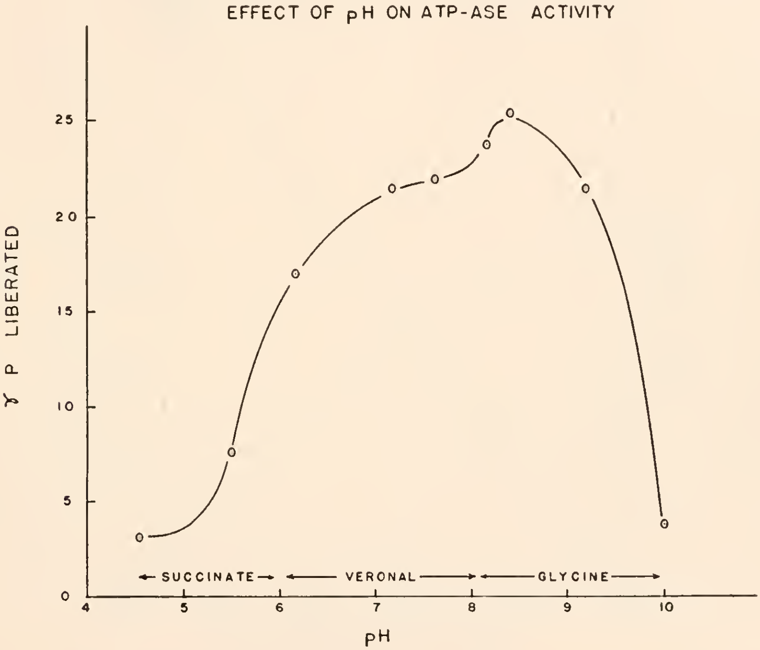

FIGURE 3. Effect of pH on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in $M/2$ KCl. Tube contents: 0.05 M KCl, 0.02 M succinate, veronal or glycine buffer, 0.02 M NaCl, 8×10^{-4} M ATP, 5×10^{-4} M $MgCl_2$, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 26° C. Ordinate—P liberated/mg. N/min., abscissa—pH.

Effect of pH. Three buffers were used covering the range from pH 4 to pH 10 in determining the optimum for liberation of phosphate from ATP by the sperm tail enzyme: succinic acid-sodium succinate, pH 4–6; HCl-sodium veronal, pH 6–9; glycine-NaOH, pH 8–10 (all measurements made with a glass electrode). In these experiments the ionic strength of the incubation medium was adjusted by the addition of appropriate amounts of NaCl. The enzyme activity rises gradually to a fairly sharp peak in both veronal and glycine buffers at about pH 8.4, and drops off precipitously to pH 10 (see Fig. 3). This is of interest inasmuch as while sperm respiration is optimal at about pH 7, and below this both motility and metabolism progressively decline, alkalinity, up to pH 8.5 and above, enhances movement (Lardy and Phillips, 1943).

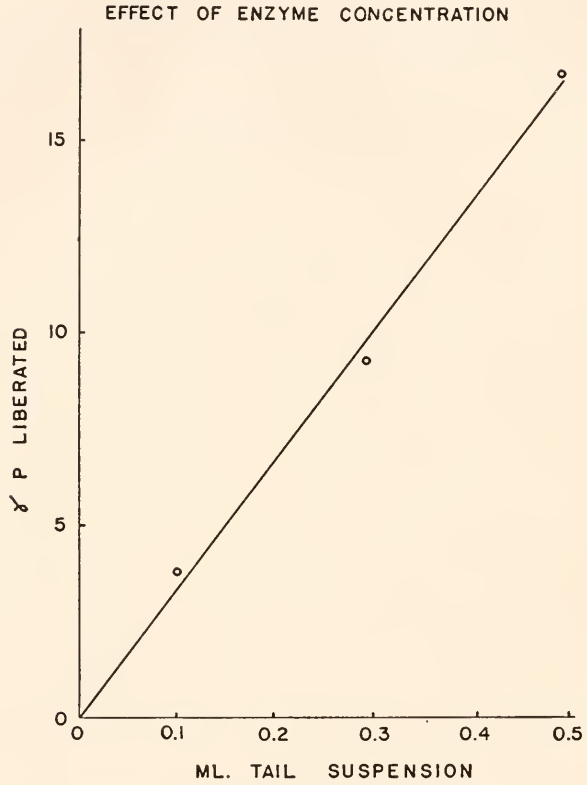

FIGURE 4. Effect of enzyme concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in $M/2$ KCl. Tube contents: 0.05 M KCl, 0.01 M veronal buffer pH 8.5, 8×10^{-4} M ATP, 5×10^{-4} M $MgCl_2$, 0.1, 0.3 or 0.5 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 26.5° C. Ordinate—P liberated; abscissa—ml. tail suspension.

Effect of enzyme and substrate concentration. The proportionality between enzyme concentration, in terms of ml. of tail suspension, and rate of dephosphorylation of ATP is shown in Figure 4. The rate of phosphorylysis may also be shown to be a function of the ATP concentration, although in higher concentrations, the relationship apparently ceases to be linear (Fig. 5). Since approximately only 50% of the labile (7′)-phosphate is liberated, even on prolonged incubation (Table I and Fig. 6), it may furthermore be concluded that the tail enzyme is a true adenosinetriphosphatase according to the definition of Steinbach and Moog (1945).

Discussion

It has previously been shown that an enzyme, concentrated in the flagellar components of spermatozoa, and either lacking or present only in minute amount in the head, is capable of dephosphorylating adenosinetriphosphate (Nelson, 1951, 1954a). Evidence has been presented establishing this as an adenosinetriphosphatase by virtue of the fact that it liberates only the terminal phosphate of ATP with appreciable velocity (Table I) under the conditions of the experiments. The fact that ATP not only plays an important role in, but is also apparently essential for, sperm motility suggests that this spermatozoan ATP-ase may have other properties in common with the ATP-ase of muscle.

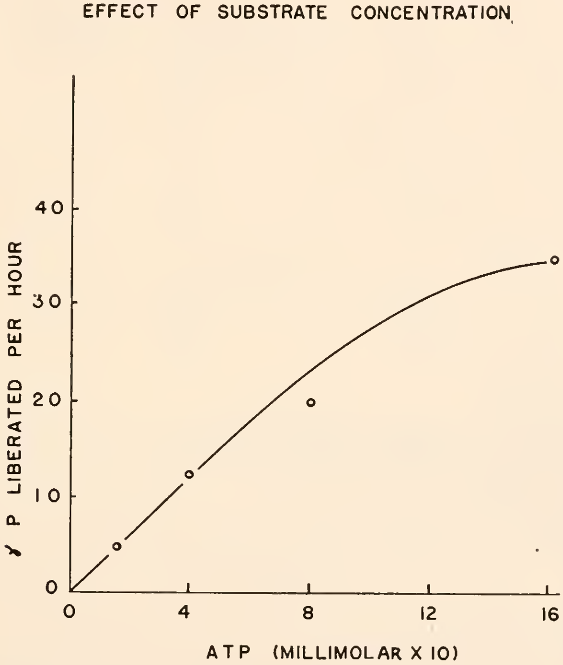

Figure 5. Effect of substrate concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M/2* KCl. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 9.2, 5×10^{-4} *M* $MgCl_2$, ATP conc. as indicated; 1.0 ml. sperm tail preparation; total 10.0 ml. Incubation: 1 hour, 27° C. Ordinate—γP liberated per hour; abscissa—ATP concentration.

The splitting of ATP by actomyosin solutions is inhibited by a wide range of Mg concentration, the degree of inhibition being a function of the Mg/Ca ratio (Braverman and Morgulis, 1948). Extruded actomyosin (myosin B) threads split ATP, the rate of phosphorylysis being enhanced by $MgCl_2$, while $CaCl_2$ caused an even greater activation (Bowen, 1952a). On the other hand, in low concentrations of ATP, $MgCl_2$ greatly accelerates, and $CaCl_2$ strongly inhibits shortening of the threads (Bowen, 1952b). Glycerol-extracted muscle fibers in 0.1 *M* KCl never reach maximum contraction in the absence of Mg ions, and the enhancing effect of calcium on the ATP-ase activity of these fibers is less than half that of magnesium, the optimum occurring at pH 7 (Sarkar, Szent-Györgyi and Varga, 1951). However, at KCl concentrations high enough to cause dissociation of the actomyosin complex in the presence of ATP, the glycerinated muscle has the same ATP-ase properties as myosin, *i.e.*, alkaline pH optimum and Ca ion activation. Calcium induces shortening when injected into isolated single muscle fibers (Heilbrunn and Wiercinski, 1947); magnesium ions are about 1/70 as potent (Woodward, 1948). Hayashi (1952) has reported that pH 7.4–7.6 is optimum for contraction of pellicular actomyosin fibers, while Kafiani and Engelhardt (1953) noted a shift in the optimum to pH 9 for pellicular myosin fiber contraction, conditions most favorable for the ATP-ase activity of actin-free myosin. It has been pointed out that pro-

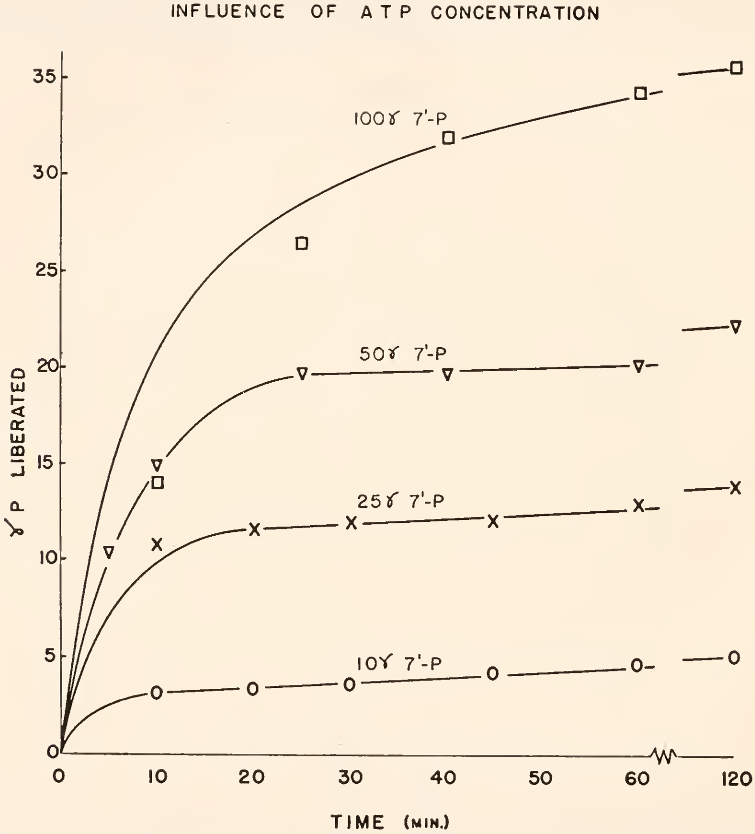

FIGURE 6. Influence of ATP concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in $M/2$ KCl. Tube contents: 0.05 M KCl, 0.01 M veronal buffer pH 9.2, 5×10^{-4} M $MgCl_2$, ATP conc. indicated as $\gamma 7'$-P, 1 ml. sperm tail preparation; total 10.0 ml. Incubation: 2 hours, 27° C. Aliquots tested at times indicated. Ordinate—γP liberated, abscissa—time in minutes.

TABLE I

Effect of substrate concentration on hydrolysis of ATP

Tube contents: NaCl 7×10^{-3} *M*, $MgCl_2$ 3×10^{-4} *M*, veronal buffer 10^{-2} *M* pH 9.2, ATP, sperm tail prep. 1 ml.; total 10 ml.; temp. 27° C.

ATP conc. mM	Time min.	% labile P hydrolyzed	Rate γP/min./mg. N
0.16	10	28.8	3.2
	30	36	1.3
	60	45	0.8
	137	50.4	0.4
0.4	10	44	12.3
	30	48.5	4.5
	60	51.2	2.4
	120	55.5	1.3
0.8	10	29.6	16.5
	25	39	8.7
	60	40.4	3.8
	120	44.0	2.1
1.6	10	14.1	15.8
	25	26.5	11.8
	60	34.4	6.4
	120	35.7	3.3

nounced calcium activation of muscle apyrase follows depression of activity occasioned by extraction of muscle with strong KCl solutions, while hydrolysis of ATP by whole muscle homogenates is not activated by calcium (Steinbach, 1947). When frozen-ground muscle extracts are dialyzed against 0.1 *M* KCl, calcium shows a strong inhibition at all concentrations while small amounts of magnesium activate ATP hydrolysis.

Thus with respect alone to the effects of pH and ion composition of the incubation medium on the activity of crude sperm tail preparations, it is difficult directly to compare the sperm tail enzyme with muscle ATP-ase. However, it may be tentatively concluded that the alkaline optimum puts the sperm tail ATP-ase in the class of myosin or of glycerinated fibers treated with strong KCl solutions, while the enzyme activation in the presence of magnesium salts is similar to the behavior of glycerinated fibers and whole muscle homogenates in weak KCl solutions. Moreover, the precipitation of the Mytilus sperm tails from KCl suspension by dilution with large volumes of water is analogous to the dilution-precipitation of myosin particles from myosin sols.

It is pertinent to consider the relationship of some of these properties of the enzyme to the behavior of the spermatozoa in response to the various factors tested. As mentioned above, the effect of pH on the ATP-ase activity of the Mytilus sperm tails seems to parallel the effects on both metabolism and motility, at least of mammalian spermatozoa. The hydrogen ion concentration of fresh samples of sea urchin semen was found to be pH 7.6–7.9 for *Arbacia punctulata*, and pH 7.5 for *Echinus esculentus* (Hayashi, 1945; Rothschild, 1948).

In some preliminary observations, it was noted that the spermatozoan ATP-ase, like that of muscle (Szent-Györgyi, 1951), is relatively indifferent to the monovalent cation, essentially the same degree of enzyme activity occurring with either K or Na solutions; although addition of K ions to a suspension of motile trout sperm in NaCl inhibits their movement (Schlenk, 1933), Mytilus sperm remain motile even after washing in isotonic KCl. Although the magnesium content of mammalian semen is only one-half to one-third of the calcium content in mg% (Mann, 1954) the omission of Mg^{++} from diluents or extenders depresses metabolism and motility, while the addition of calcium adversely affects motility, glycolysis and respiration (Lardy and Phillips, 1943).

Summary

1. The tails of the sperm of *Mytilus edulis* dephosphorylate ATP.

2. $MgCl_2$ (5×10^{-4} *M*) increases the rate of the enzyme activity in 0.02 *M* KCl to more than double that of the control; the same concentration of $CaCl_2$ causes about 25% greater activity than the control.

3. In appropriate concentrations magnesium potentiates the effect of calcium, but all concentrations of calcium tested depress the magnesium activation.

4. The enzyme activity rises gradually to a fairly sharp peak at pH 8.4 in veronal and glycine buffers and drops off abruptly to pH 10.

5. The rate of dephosphorylation of ATP is proportional to the enzyme concentration.

6. Phosphorylysis is also a function of the ATP concentration, except at higher levels of ATP.

7. This is apparently a true ATP-ase since only about 50% of the labile phosphate is liberated with appreciable velocity from ATP.

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INFECTION OF COCKROACHES WITH HERPOMYCES (LABOULBENIALES). IV. DEVELOPMENT OF H. STYLOPYGAE SPEGAZZINI [1, 2]

A. GLENN RICHARDS AND MYRTLE N. SMITH

Department of Entomology and Economic Zoology, University of Minnesota, St. Paul 1, Minnesota

Although some 1500 species of Laboulbeniales have been described, the development of only a few species of relatively simple structure has been worked out in detail (Benjamin and Shanor, 1950). For the genus *Herpomyces,* Thaxter (1931) gives a short description of the general features of development, presumably pieced together from stages noted during the description of some two dozen species. But nowhere is there an illustrated description of the development of any species of *Herpomyces,* or, indeed, of any member of the Laboulbeniales showing this much structural complexity. The present paper gives a pictorial presentation of the development of both female and male plants with special reference to the differences when growth is on setae in contrast to the general antennal surface, and to the origin of multiple perithecia and development of the peculiar basal "shield" covering the secondary receptacle in this species.

In contrast to *Stigmatomyces* (Thaxter, 1896) and *Laboulbenia* (Benjamin and Shanor, 1950), the development of *Herpomyces stylopygae* is less precise. There is more variation in relative cell sizes and absolute cell number. The general picture, however, follows a regular pattern of development determined by sex and location on the host. And, in contrast to the dioecious *Laboulbenia formicarum,* the cell lineage in male and female plants is entirely different instead of nearly the same up to the time of differentiation of the reproductive organs.

Materials and Methods

Many of the specimens in our colony of the oriental cockroach (*Blatta orientalis* L.) are infected with *H. stylopygae,* and the fungus continues to maintain itself in these colonies. Since the antennal infections can be seen with the unaided eye, infected individuals were caught, the antennae amputated, prepared as whole mounts in lacto-phenol plus cotton blue, and examined. For older stages it is convenient to scrape the antennae and then study the dislodged plants floating freely in the stain medium. Younger stages have to be examined on the surface of whole antennae or on setae.

Serial sections of infected antennae were available from the study presented in Part 2 of this series; they were useful for certain points in later development when

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[2] The work reported in this paper was done in connection with a contract between the Office of the Surgeon General, U. S. Army, and the University of Minnesota.

the plant had become too large for satisfactory examination as a whole mount (Figs. 26–29, 44).

The method of drawing particular stages and then attempting to seriate the drawings has distinct limitations. Yet it is not feasible to follow the development of a single plant when they can be grown only on living cockroaches and require about two weeks for maturation. Most points seem logically satisfactory but a few remain ambiguous.

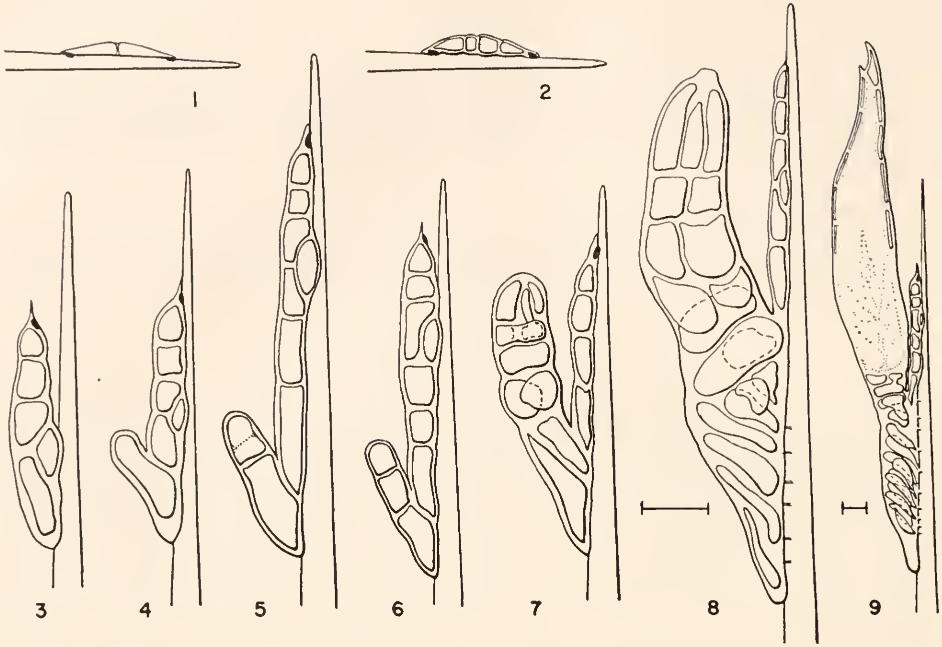
The structure of haustoria is omitted (see Richards and Smith, 1955b).

All drawings were made to scale free-hand using an ocular grid and ruled paper. Certain stages have been illustrated by photographs in preceding parts of this series; such photographs are referred to at the appropriate places in the present paper.

Development of the Female Plant

The spores are released from the asci within the perithecia (photographs in Richards and Smith, 1955a). At the time of ejection and transfer they are in the 2-cell stage (Fig. 1) but soon become 4-celled. In size they range from 2–3 μ in width and 20–30 μ in length. In the 2-cell stage the two cells are of about the same size and make a symmetrical spore, but in the 4-cell stage the cells may appear similar or quite dissimilar with no particular significance evident for the differences (Figs. 10–14). Many spores do not develop beyond this stage, or at least do not do so promptly (Richards and Smith, 1955a). While spores occasionally develop on other parts of the body, almost all of the mature plants of *H. stylopygae* are found on the antennae of the host. We did not find any way to distinguish spores that would develop into male plants from those that would develop into female plants until differentiation subsequent to the 4-cell stage.

On setae the first evidence of further growth is a considerable increase in volume, although this increase does not seem to be a constant amount and is not necessarily as great as the differences between Figures 2 and 3. Beyond this initial change in volume, the first two cells and the fourth cell do not change further. Up to this stage there has been no apparent difference between the two ends of the spore, but with growth proceeding basally the two ends become obviously different (Fig. 3). The base of the plant is always towards the base of the seta, but it is not known whether this is due to orientation being produced by orientation on the host or whether only those spores so oriented develop (see Richards and Smith, 1955a). The third or subbasal cell grows basally over the fourth or basal cell, and divides repeatedly to give a row of three to five cells. The terminal cell bulges away from the seta (Fig. 4) and divides to give two cells (Fig. 5). The apical one of these promptly divides to give a third cell (Fig. 6). At this stage, then, we have a row of five to seven cells which will not develop further (the primary receptacle), and a basal obliquely protruding group of three cells which will form the remainder of the plant. The basal of these three cells divides repeatedly extending the plant down the shaft of the seta (Figs. 7–9) to form the secondary receptacle, processes from individual cells penetrating as minute haustoria into the shaft of the seta (photographs in Richards and Smith, 1955b). The middle one of the row of three cells divides to give the basal cells of the perithecium, and the apical one of the three cells divides first transversely, then longitudinally to develop into the peri-



Figures 1–9. Development of female plant of *H. stylopygae* growing on antennal setae of the oriental cockroach. Figures 1–8 at same magnification, Figure 9 smaller, the bars representing 10 μ. See text for explanation.

thecium (see below). This cell lineage is diagrammed as the top sequence in Figure 55.

When growing on setae, the characteristic structure shown in Figure 9 is produced. Only the perithecium resembles that of plants growing on the general surface of the antenna. The secondary receptacle is not only of grossly different shape but its component cells are also different, and there is no trace of development of the peculiar "shield." In some cases, a spore germinating near the base of a seta will reach the base and spread across the antennal surface (this is common for the species *H. ectobiae*); in these cases the plant changes more or less from the growth pattern on setae to that on the general body wall of the antenna.

Female plants growing on setae are usually alone and usually have only a single perithecium. Sometimes one finds both a male and a female plant growing together on a seta. And, occasionally, a pair of female plants are found alongside one another on a single seta—it is not known whether these arise from one spore or two.

On the general antennal surface (*i.e.,* not on setae) the pattern of development of female plants is more complex (Figs. 15–36). Development begins as on setae by swelling of the 4-cell stage and growth of the third (subbasal) cells in the basal direction (Fig. 15). The prolongation of the third cell becomes constricted off (Figs. 17, 18) and immediately develops as a protrusion which divides to give two cells, the more apical one soon dividing to give two, then three, cells (Fig. 20). The subsequent history of this protruding row of cells is similar to that of the protruding basal row of Figure 6. The apical one eventually gives rise to the perithecium, the middle one gives rise to the basal cells of the perithecium, and the basal one divides longitudinally (Figs. 21–24) to give rise to a basal row of five to six elongated cells (Fig. 25) which forms the secondary receptacle (and sends a large haustorium penetrating through the insect's cuticle into the underlying epidermis; see Richards and Smith, 1955b).

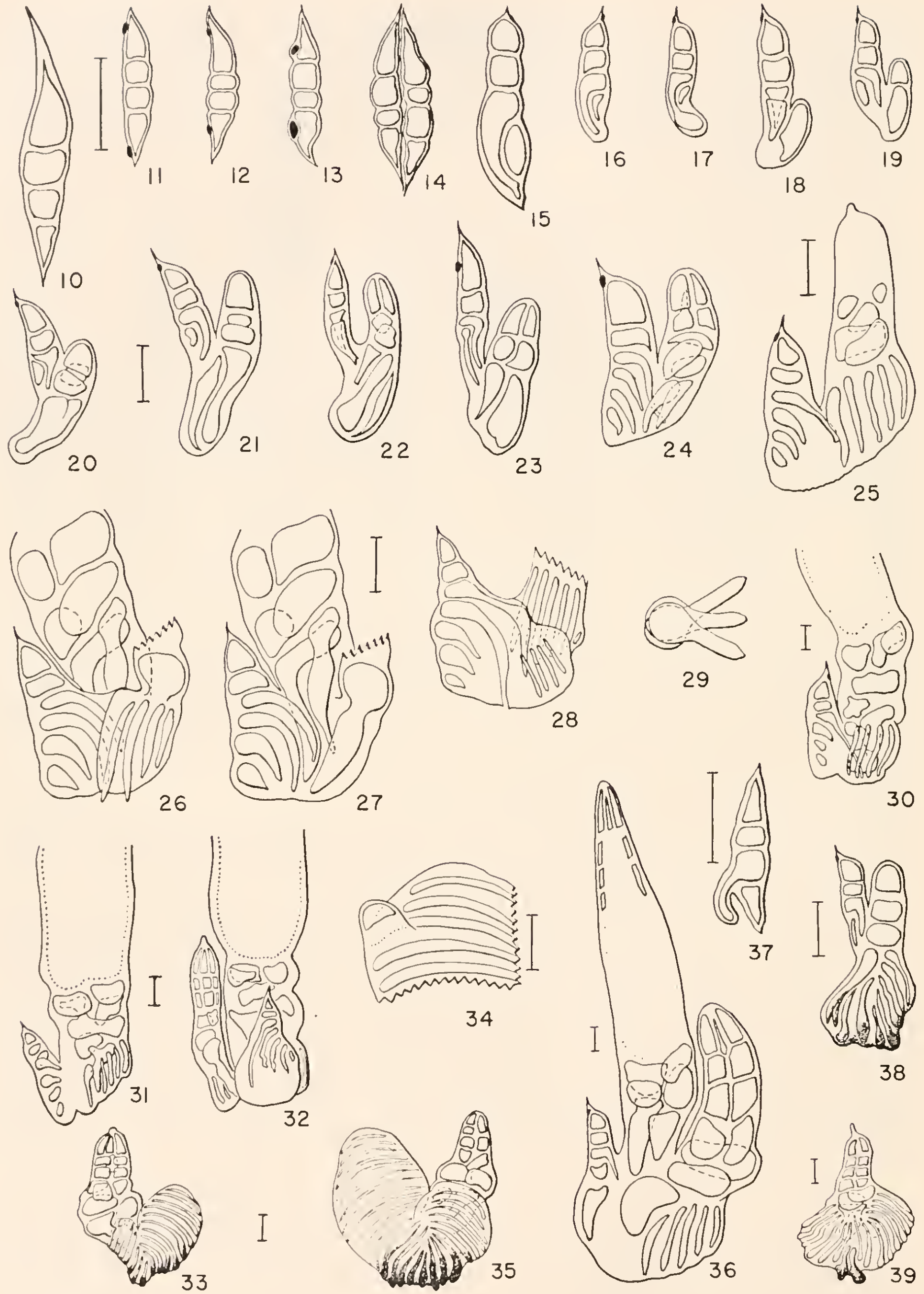
But while the above development is taking place, the original group of four cells (Figs. 16–23) increases to six (Fig. 24) or occasionally seven (Figs. 28, 32), and comes to constitute a distinct portion of the plant (called the primary receptacle). It is uncertain which cells divide to give this increase. In Figures 15–17 it seems that the fourth cell is being by-passed (as on setae) but later it seems to develop (Figs. 18, 22) and to form a projection similar to that from the third cell (Fig. 23). With the increase in cells of the primary receptacle to six, it is found that the third, fourth and fifth all have long processes (Figs. 26–28). These processes do not lie in the same plane but diverge in the manner diagrammed in Figure 29 (which is reconstructed by focal plane analysis of the section drawn in Figure 28; it represents Figure 28 as it would appear if viewed along the plane of the page). Each of these processes buds off a cell which divides and gives rise to a secondary receptacle and a perithecium. By this stage the structure is too complicated for satisfactory analysis from whole mounts. Sections such as those drawn in Figures 26–28 show the process from the third cell extending to the base of the oldest perithecium, the process from the fourth cell extending to the base of a second and much younger perithecium (as Figs. 32 and 36), and the process from the fifth cell extending to a still younger perithecium. (Figures 26 and 27 are drawn from the same section, the base of the secondary receptacle of the younger perithecium being omitted from Figure 27 to permit showing underlying cells.) This cell lineage is diagrammed as the middle sequence in Figure 55.

After budding off of cells to form secondary receptacles and their perithecia (two secondary receptacles shown in Fig. 28), the primary receptacle remains static but can be found underneath the mature plant.

After several perithecia are underway, the secondary receptacle of one of them, seemingly that of the oldest perithecium, begins to hypertrophy. A lateral outgrowth develops involving an increase in the number of elongated cells of the secondary receptacle and a turning of these new cells so that they extend transverse to the plant's axis rather than parallel to it (Fig. 33). Or such growths may develop on both sides (Figs. 35, 47). This growth gives rise to the peculiar "shield" covering the base of the plant (but found only in *H. stylopygae* and *H. periplanetae*). Outgrowths from both sides can give rise to paired "shields" (Fig. 35). Sometimes the developing "shields" themselves grow little additional lobes (Fig. 34) but such are not common.

Shortly after the "shield" begins to grow it begins to blacken basally in *H. stylopygae* (not in *H. periplanetae*) as shown by stippling in Figure 33. Later the basal blackening becomes more extensive (Fig. 35). This darkening is a chemical process showing histochemical reactions similar to those of the host's cuticle during sclerotization (Richards, 1954).

Occasional plants are found which do not fit into the above sequence. Sometimes germinating spores develop in the manner shown in Figure 37; this type of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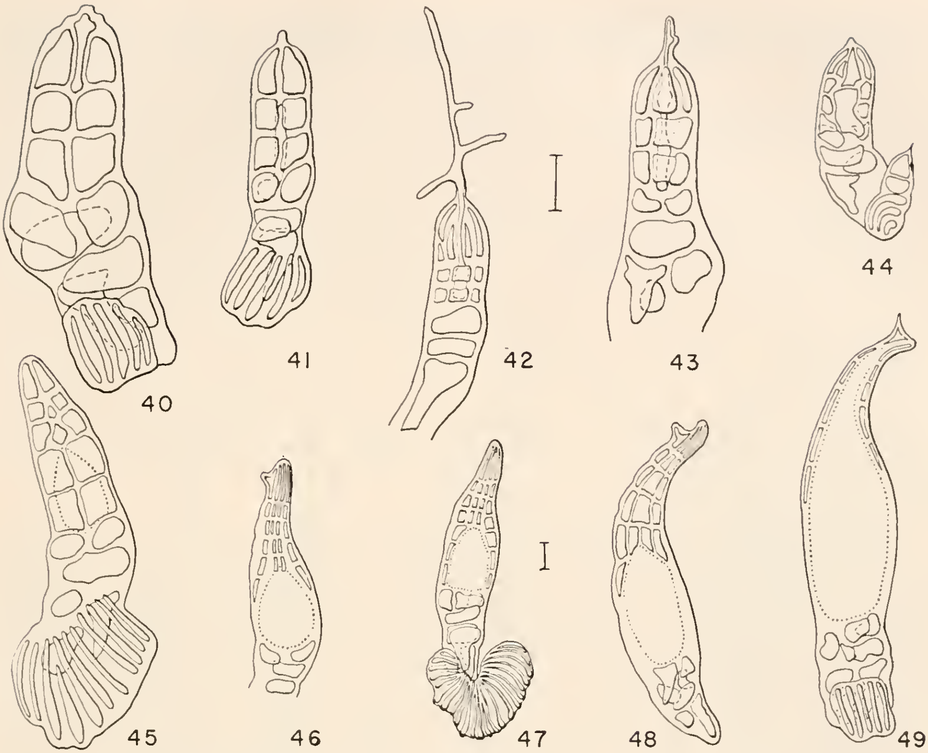
Figures 10–39. Development of female plant of *H. stylopygae* growing on general surface (not on setae) of antennae of the oriental cockroach, with particular reference to the basal receptacles and "shield." See text for explanation. Drawn with primary receptacle to the



Figures 40–49. Development of the perithecium of female plants of *H. stylopygae* from stages equivalent to Figures 8 and 25 to maturity. See text for explanation. Magnifications: vertical bars 10 μ (Figs. 40–45 at same magnification, Figs. 46–49 at same).

outgrowth is the common expression of incipient germination on greased collodion membranes but is rare on antennae. We have no idea whether or not such spores can continue development on antennae. Occasional plants show increase in number of elongated cells of the secondary receptacle, either irregularly (Fig. 38) or regularly (Fig. 45) or symmetrically (Fig. 39). Too few such cases were seen for us to have any idea of the subsequent development of such plants.

After their initiation, the development of perithecia is similar for plants growing on setae and on the general antennal surface. The apical cell cut off by the first division of the basal cell of Figures 4 and 18 divides transversely. The row of three cells formed (Figs. 6, 20) gives rise, respectively, to the secondary receptacle (plus "shield"), the basal cells below the perithecium, and the perithecium, as already mentioned. The middle one of the original row of three divides more or less irregularly to give rise to a group of relatively large cells of various shapes (Figs. 26, 30, 40–44). These basal cells of the perithecium are multinucleate, and some

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left, secondary receptacle and perithecia to the right except for Figure 32 where the primary receptacle is uppermost (young perithecium on left) and Figures 33, 35 and 39 which do not show a primary receptacle. Magnifications: all vertical bars represent 10 μ and refer to adjacent sketches (Figs. 10–15 at same magnification, Figs. 16–29 at same).

or all of them are interconnected by cytoplasmic strands and bridges (photographs in Richards and Smith, 1955b). The visible cytoplasmic connections are limited to this group of cells; they do not extend on into the secondary receptacle basally or into the perithecium distally. The apical cell of the original row of three first divides longitudinally (Figs. 22–24) and then transversely to give rise to a row of three or four sets of four cells each (Figs. 8, 40). At first these are not distinctly set off from the cells that will form the basal cells of the perithecium, but with further divisions giving smaller cells (Figs. 42, 43) the distinction becomes obvious. At the stage shown in Figure 41, one of the basal cells of this group becomes displaced to the center and develops a process which grows distally. This process reaches and grows through the tip of the perithecium to form an elongated (up to 43 μ) and variously branched trichogyne (Fig. 42). No two trichogynes have the same appearance. Soon the trichogynes become more or less covered with spermatia (not shown in drawing) from male plants. With fertilization accomplished, the trichogynes begin to regress while the portion in the center of the developing perithecium enlarges to form a distinct ascogonium. The ascogonium is shown in Figure 43 by a dotted line, but its size and distinctness are more clearly seen in serial sections through the plant (Fig. 44).

After fertilization and regression of the trichogyne, the young perithecium begins to elongate (Fig. 45) and there is seen a differentiation of several rows of smaller cells apically contrasting with larger cells surrounding the swelling ascogonium. Subsequent divisions increase the number of smaller apical cells as the perithecium continues to elongate (Figs. 46–49); these cells form the apical portion of the perithecium including the canal through which spores later make their exit. Simultaneously, the ascogonium swells enormously (Figs. 46–49) stretching the surrounding cells into a thin surface sheet. Details of ascogenesis within the ascogonium were not worked out because of the poor visibility in whole mounts and the lack of a sufficient number of early and intermediate stages in our serial sections. Clearly, however, by the stages shown in Figures 9 and 49, *i.e.,* by the time the perithecium has developed fully the characteristics used by taxonomists for identification of species, asci have developed.

The first perithecium developed on multiperithecial plants reaches maturity (presumably including functional maturity) while the second and third perithecia are still quite young (Figs. 32, 36). Later all the perithecia become mature, and the infected antennae show the customary appearance of plants with perithecia all of which are mature (photographs in Richards and Smith, 1955a, 1955b). One has to have infections including relatively young plants to obtain specimens with perithecia of obviously different ages.

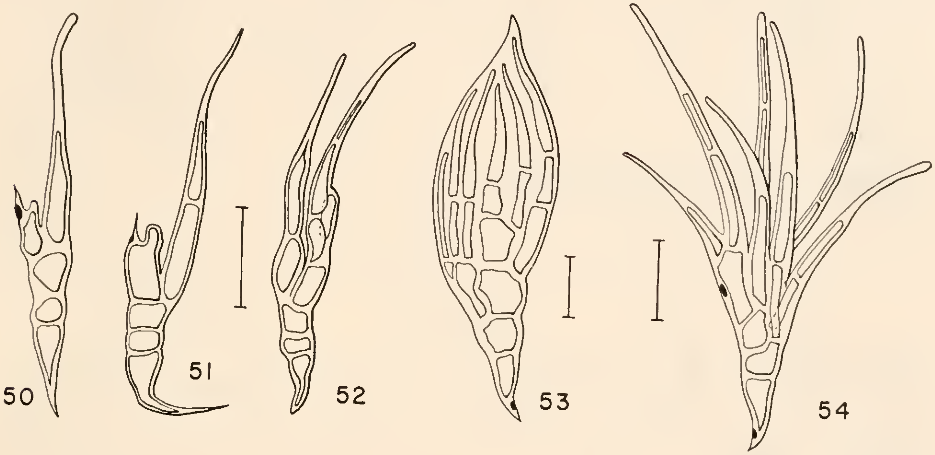
Development of the Male Plant

Male plants of *H. stylopygae* seem to be considerably less common than female. In contrast to the female plant, the male is very simple. It also appears to develop in the same manner whether on setae or on the general antennal surface (but is relatively seldom seen on setae in this species).

The 4-cell spore of the male plant attaches to the insect's cuticle by the basal cell (with a small haustorium penetrating therefrom; see Richards and Smith, 1955b), and the apical cell begins to bud off antheridia (Fig. 50). These appear to be budded off one at a time (Fig. 51) until a considerable tuft has developed (Figs. 53, 54). The mature male plant, then, consists of a stalk formed from the basal three cells and a tuft of antheridia budded off the apical cell. The cell lineage is diagrammed as the bottom sequence in Figure 55.

The antheridia initially consist of a single elongated cell (Fig. 50) but this soon divides to give a linear array of two or three cells with a tip and duct extending farther (Figs. 51–54). The spermatia produced (not drawn) are small blobs less than a micron in each dimension (seem to average about 0.5 × 0.8 μ).

In some cases the tuft of developing antheridia is encased in a sheath (Fig. 53). In other cases no such sheath is evident. At maturity the tuft presents a picture similar to Figure 54 or with a few more antheridia.



Figures 50–54. Development of male plant of *H. stylopygae* on antennae of the oriental cockroach. See text for explanation. Magnifications: vertical bars 10 μ.

Discussion

Despite the limitations imposed by the method of seriating developmental stages found in different specimens, it has been possible to work out a logical sequence for most of the ontogenetic details. Of most general interest is the difference in cell lineage for male and female plants, and for female plants growing on setae in contrast to ones growing on the general antennal surface. These differences are summarized in Figure 55 where "A" and "B" represent the cells of the 2-cell spore giving rise respectively to cells "A_1," "A_2" and "B_1," "B_2" of the 4-cell spore.

In the female plant growing on setae, the 4-cell spore develops into a row of six or seven cells by repeated division of the third or subbasal cell (B_1). The most basal one of this row (B_{1b} or B_{1c} depending on whether 6 or 7 cells) differentiates into a cell (C) which divides to give basally a cell (D) which produces a secondary receptacle (SR) and haustorium (H), and obliquely apically a cell (E) which produces a perithecium (P). Cells "A_1" to the next to the last of the "B" series remain as the so-called primary receptacle. No "shield" is developed.

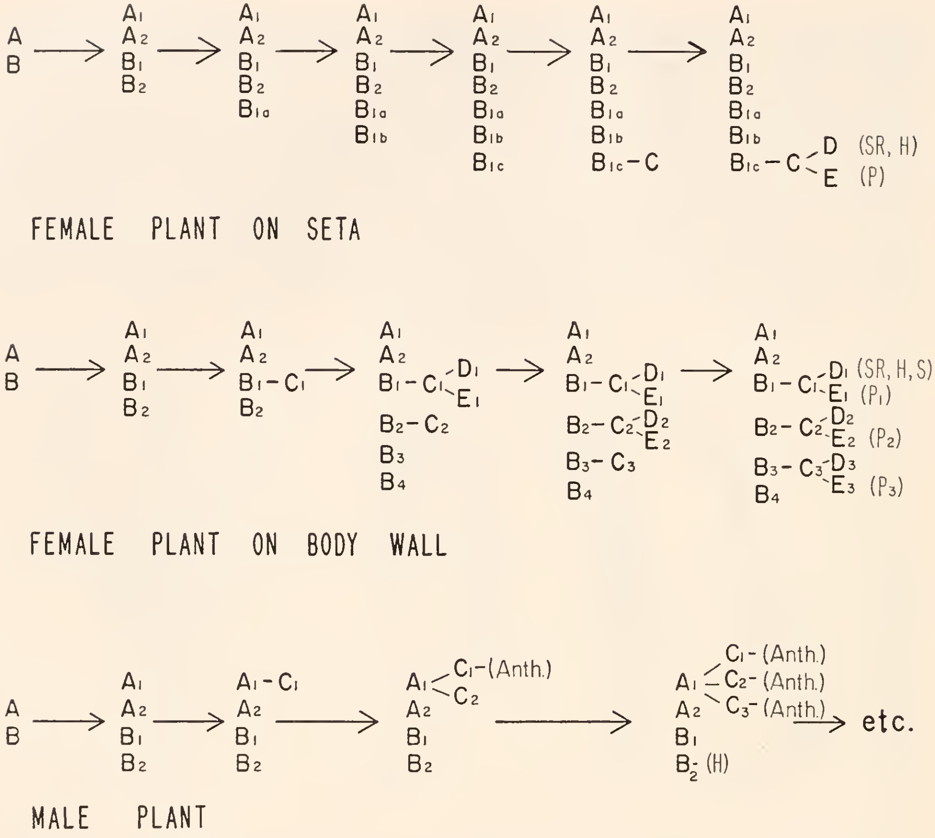

A
B
A1 A2 B1 B2
A1 A2 B1 B2 B1a
A1 A2 B1 B2 B1a B1b
A1 A2 B1 B2 B1a B1b B1c
A1 A2 B1 B2 B1a B1b B1c-C
A1 A2 B1 B2 B1a B1b B1c-C D (SR, H) E (P)
FEMALE PLANT ON SETA
A1 A2 B1-C1 B2
A1 A2 B1-C1 D1 E1 B2-C2 B3 B4
A1 A2 B1-C1 D1 E1 B2-C2 D2 E2 B3-C3 B4
A1 A2 B1-C1 D1 (SR, H, S) E1 (P1) B2-C2 D2 E2 (P2) B3-C3 D3 E3 (P3) B4
FEMALE PLANT ON BODY WALL
A1-C1 A2 B1 B2
A1 C1-(Anth.) C2 A2 B1 B2
A1 C1-(Anth.) C2-(Anth.) C3-(Anth.) A2 B1 B2 (H)
etc.
MALE PLANT

FIGURE 55. Diagrams of cell lineage in developing plants of *H. stylopygae*. See text for explanation.

In the female plant growing on the body wall proper of the antenna, the third or subbasal cell (B_1) produces by budding a cell (C_1) which in turn gives rise to two cells (D_1 and E_1) which produce the secondary receptacle (SR) and perithecium (P), respectively. But, simultaneously some of the "B" cell series have divided to give additional cells "B_3" and "B_4" (and sometimes a "B_5"). Cell "B_2" then differentiates in a manner similar to that of "B_1," and later cell "B_3" does likewise. When seven cells are present instead of six it seems that cell "B_4" also does likewise at a still later time. Cells "A_1," "A_2," and the last one of the "B" series contribute nothing except that the whole of the "A" and "B" series, after budding off of the "C" series, remain as the so-called primary receptacle. And, finally, the first secondary receptacle (SR) gives rise to the single large haustorium (H) and then later hypertrophies to produce the peculiar "shield" (S) covering the base of the plant.

In the male plant, the basal cell (B_2) forms the attachment to the host and sends a haustorium (H) into the insect, while the apical cell (A_1) buds off cells (the "C" series) each of which gives rise to an antheridium (Anth.).

Clearly, in the female plant the "A" series contributes nothing (except for remaining as part of the primary receptacle) and the whole visible plant develops from the "B" series, perhaps just from the subbasal cell (B_1), whereas in the male plant attachment is by the basal cell (B_2) and antheridia develop from the apical cell (A_1). Cell lineage is, then, fundamentally different in the two sexes.

The two types of growth of female plants seem to us merely products of growth restriction. In both types growth is initiated by the subbasal cell (B_1). Presumably growth on the general surface of the antenna is unrestricted. Several of the cells of the "B" series then bud off one after another processes which give rise to secondary receptacles and perithecia. The oldest of the secondary receptacles later hypertrophies to give the peculiar growth called the "shield." On a seta the secondary receptacle either has to protrude laterally into space or grow along the seta —it grows along the setal shaft. Rationalizing, once the terminal cell has begun to form a receptacle and perithecium, there simply is no place for another cell of the "B" series to develop unless it moved around the seta (which it at least usually does not do although some paired plants seen on setae could be interpreted as having arisen in this manner). On both body wall and setae the secondary receptacles hypertrophy later in development; on the body wall this growth gives the "shield," on setae it gives an elongation of the secondary receptacle with increasing cell number. It seems to us that female plants of *H. stylopygae* develop on an essentially similar pattern in both cases but show considerable plasticity to fit the geometric limitations of the particular surface on which they are growing.

SUMMARY

The development of male and female plants of the dioecious fungus *Herpomyces stylopygae* on cockroach antennae is described. Cell lineage is fundamentally different for male and female plants. The gross structure and apparent cell lineage are different for female plants growing on setae in contrast to ones on the body wall. An interpretation in terms of developmental plasticity and geometric limitations of the host's surface is suggested for the two types of growth of female plants.

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DEVELOPMENTAL MODIFICATIONS IN THE SAND DOLLAR CAUSED BY ZINC CHLORIDE AND PREVENTED BY GLUTATHIONE[1]

OLIN RULON

Dept. of Biological Sciences, Northwestern University, Evanston, Illinois, and the Hopkins Marine Station, Pacific Grove, Calif.

Problems dealing with growth and differentiation are of obvious importance. The modification and control of cleavage rate, developmental patterns and differentiation in echinoderms by the use of chemical agents has greatly interested the writer for several years. In 1947, work was begun which involved testing the effects of various enzyme inhibitors and activators on cleavage and developmental patterns of the early larva. Many agents, which reputedly affect one or more enzymes or enzyme systems, have been used. As the work has progressed the problem has become increasingly more complex. At the present moment it appears that the term "specific" as applied to morphological changes from enzyme inhibition or activation is a relative term. Granted that a particular agent may affect a certain enzyme specifically, the effect on developmental pattern may be quite similar to the effect produced by another agent on another enzyme, provided both enzymes are in the complexity of interactions leading to a particular modification of structure. Most enzyme inhibitors affect more than one enzyme. Many have multiple effects. Some activate one enzyme while inhibiting another. Concentrations of inhibitors are extremely important. Some inhibitors affect many enzymes in high concentrations but in lower concentrations only certain of the enzymes.

Despite the extreme complexity of the problem certain effects of enzyme inhibitors on development have become increasingly clear. Totally different agents may cause the same modification in developmental pattern but modifications may differ widely between various groups of inhibitors. One group of agents may be particularly effective in causing ectodermization while another may cause exogastrulation and entodermization almost 100 per cent of the time if concentrations and exposure periods are controlled. Some agents have little or no effect on the gastrulation process but will cause the embryo to develop around a radial symmetry. Some agents affect the differentiation of skeletal spicules more than other agents. It has recently been found (Rulon, 1952, 1953a, 1953b) that selenium, cobalt, and nickel are all effective in causing the development of radial embryos which have elongated in the polar direction and differentiated with respect to a new pattern. At present our knowledge is extremely limited on many of the chemical, physical, and biological factors in development. This author is making an attempt to correlate certain morphological changes with chemical action. He believes that with data from a large number of experiments involving the use of enzyme inhibitors and activators, and with morphological changes as markers, it should be possible to determine certain common denominators between developmental changes, enzyme activity, enzymogenesis, histogenesis, etc.

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[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, director of the Hopkins Marine Station, Pacific Grove, California, for many considerations.

The present report deals chiefly with experiments in which only one agent was used. Zinc chloride was chosen as the test agent because of its known action on certain enzymes. Histidase is inactivated while prolidase, carnosinase, certain dipeptidases, alkaline phosphatase, and pyrophosphatase I are activated by the zinc ion (see Sumner and Somers, 1953, for references). Zinc is also known to react with sulfhydryl groups (Potter and DuBois, 1943) and it is known that the sulfhydryl radical is essential to the activity of many, but not all, enzymes (Sumner and Somers, 1953).

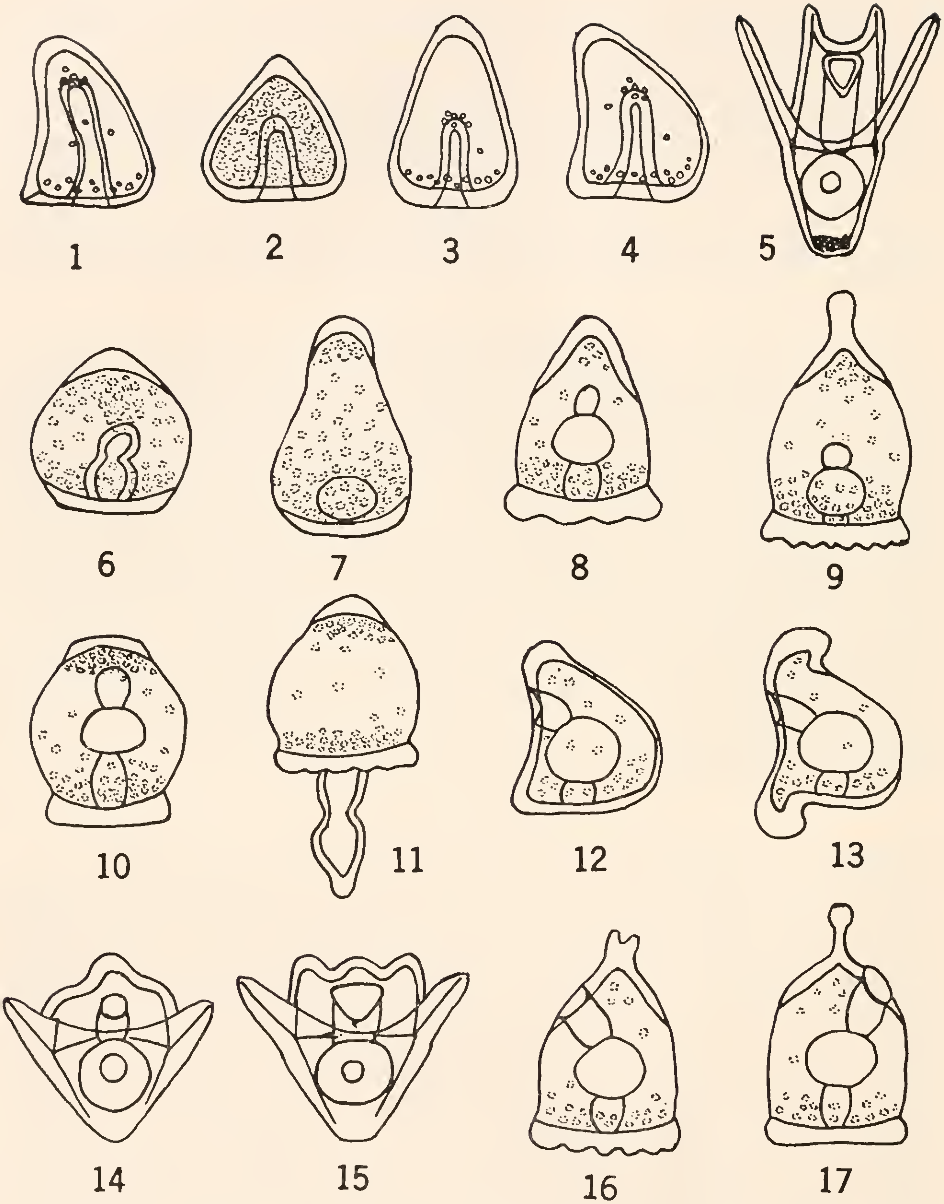
Material and Methods

The adult sand dollars (*Dendraster excentricus*) used in this study were dredged from Monterey Bay near the Hopkins Marine Station, Pacific Grove, California, during the summers of 1953–54. They were maintained in running sea water in the laboratory. Ripe eggs were removed from mature females by cutting away the oral surface of the test and allowing them to exude from the ovaries. A medicine dropper was used to transfer the eggs to sea water where they were washed several times. Fertilization was accomplished by adding a few drops of sperm suspension. All experiments were performed in the aquarium room of the Station where the temperature varied by no more than one degree from 18 degrees C. All eggs were kept in uncrowded lots in finger bowls and out of direct sunlight. Smoking was not permitted in the room. In preliminary experiments, lots of eggs were placed in sea water solutions of zinc chloride ranging from $M/100$ to $M/320,000$. Since the higher concentrations were very toxic only experiments using concentrations ranging from $M/10,000$ to $M/320,000$ are reported here.

Experimental

1. *Continuous exposure to zinc chloride beginning with the one-cell stage*

When newly fertilized eggs were placed directly into $M/10,000$–$M/20,000$ solutions of zinc chloride the rate of cleavage was slightly inhibited as noted by observations of the six-hour blastula. No such inhibition was seen in $M/40,000$–$M/320,000$ solutions. After 24 hours, when the controls were normal preplutei (Fig. 1), the larvae in $M/10,000$ were chiefly slow-moving, cell-filled, radial, inhibited gastrulae. Approximately 50 per cent were stunted, conical forms (Fig. 2) and a few showed slight bilaterality. In $M/20,000$, the larvae were almost all conical gastrulae with fewer internal cells. Roughly 50 per cent were free-swimming and there was an occasional exogastrula. Those in $M/40,000$ were chiefly free-swimming conical types with about 25 per cent exogastrulation. Most of the larvae in $M/80,000$ were slightly elongated, radial, conical gastrulae (Fig. 3) with basal regions slightly thicker than those of larvae developing in higher concentrations. An occasional bilateral form had developed in this solution and approximately 25 per cent had undergone exogastrulation. At 24 hours, $M/160,000$, gave about 75 per cent slightly bilateral gastrulae and 25 per cent exogastrulae. All the embryos in $M/320,000$ were late bilateral gastrulae (Fig. 4) that were not far behind the controls.



FIGURES 1–17. Figure 1, normal 24-hour prepluteus. Figures 2–4, 24-hour larvae exposed since fertilization to *M*/10,000 (Fig. 2), *M*/80,000 (Fig. 3) and *M*/320,000 (Fig. 4) solutions of zinc chloride. Figure 5, normal 48-hour pluteus. Figures 6–15, 48-hour larvae exposed since fertilization to *M*/10,000 (Figs. 6–7), *M*/20,000 (Fig. 8), *M*/40,000 (Figs. 9–11), *M*/160,000 (Figs. 12–14), and *M*/320,000 (Fig. 15) solutions of zinc chloride. Figures 16–17, 48-hour larvae exposed since the 6-hour blastula stage to *M*/80,000 zinc chloride.

After 48 hours, when the controls were well-developed, bilateral plutei (Fig. 5), the larvae in *M*/10,000 were radial, cell-filled, slow-moving gastrulae (Figs. 6, 7) with many slightly elongated in the polar direction. In *M*/20,000 the picture differed slightly in that the apical thickening was usually more pronounced, the enteron was better developed, and the basal region was commonly thickened into a fluted, ciliated band (Fig. 8). There was approximately 10 per cent exogastrulation. In *M*/40,000, all had radial symmetry, basal ciliated bands, and apical thickenings which often extended upward as buds of ectoderm (Figs. 9, 10). Here, also, approximately 10 per cent showed exogastrulation (Fig. 11). In *M*/80,000 the situation was not much different but in *M*/160,000 most of the larvae were bilateral, without arms, and with little or no skeleton (Fig. 12). Commonly a ventral ciliated band was over-developed (Fig. 13). There were a few radials but some had developed into recognizable plutei (Fig. 14). In *M*/320,000 about 50 per cent were still bilateral forms without arms or skeleton while the remaining 50 per cent graded up to wide-angled plutei with wide, but poorly differentiated, oral lobes (Fig. 15).

These experiments, dealing with the continuous exposure of newly fertilized eggs to various concentrations of zinc chloride, show that high concentrations inhibit cleavage but that lower concentrations, which have no noticeable effect on cell division, do cause marked changes in developmental pattern. The chief effect of strong solutions is in causing the development of radial, poorly-differentiated larvae. As the strength of the solution was decreased, thickened ciliated basal bands appeared and the larvae elongated in the polar direction. In the weaker solutions bilaterality developed and in the weakest, plutei with increased ventral areas but with poorly differentiated oral lobes were found. A slight bilaterality, that was present at 24 hours in M/10,000, had disappeared by 48 hours. Lots showing 25 per cent exogastrulation at 24 hours showed only 10 per cent of such development at 48 hours. Skeleton was completely inhibited in all concentrations above *M*/80,000 and in solutions as low as *M*/320,000 only 50 per cent of the larvae showed any skeletal development.

2. *Continuous exposure to zinc chloride beginning with the mid-blastula stage*

When 6-hour blastulae were placed in *M*/10,000 zinc chloride solution all had developed, after 18 hours, into radial, conical forms (as in Figs. 2, 3). Most were free-swimming, a few had an excess of internal cells and there was only an occasional exogastrula. In *M*/20,000 and *M*/40,000 radial, conical forms had developed but in the case of the lower concentration the gut was usually better differentiated and commonly bent toward one side. In *M*/80,000 most were still free-swimming conical gastrulae but slight bilaterality could be seen in a few. It was also noted that many were irregular around their basal regions as if fluted circles of cilia were developing. Approximately 10 per cent of the larvae had undergone exogastrulation in this solution. In *M*/160,000 and *M*/320,000 the larvae had become bilateral (as in Fig. 4) and those in the weakest solution showed a trace of skeleton.

At 48 hours the larvae, that had been in *M*/10,000 zinc chloride since the 6-hour blastula stage, were not very different from those that had been in this concentration since fertilization. That is, they were slow-moving, radial forms with an excess of internal cells (as Figs. 6, 7). In *M*/20,000 and *M*/40,000 the blastulae had developed in a manner similar to those exposed from the one-cell stage to comparable solutions except that with the blastulae, very few exogastrulae appeared. The situation in *M*/80,000 was not far different except that here a well-developed enteron was the rule and commonly a stomodaeum developed (Figs. 16, 17). In *M*/160,000, at least 50 per cent showed bilaterality and graded into plutei possessing short anal arms with skeletal spicules. The remainder resembled those from higher concentrations. With *M*/320,000, practically all were inhibited, bilateral plutei with short anal arms at various angles, asymmetrical, or askew and a few with widened, but poorly differentiated, oral lobes.

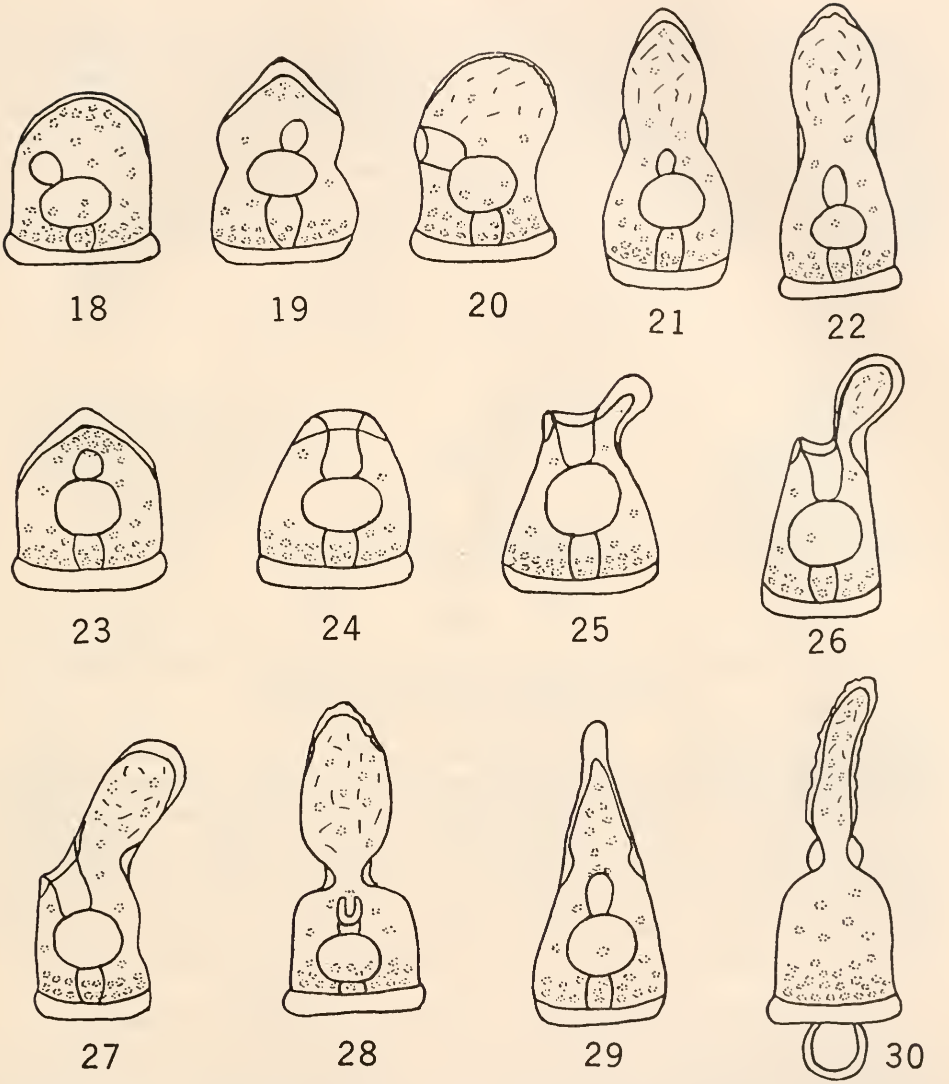
The data from the above experiments show that treatment with zinc chloride after cleavage gives larvae that are only slightly less modified than those exposed continuously after fertilization. The chief differences were that fewer exogastrulae developed, skeleton differentiated in slightly higher concentrations, and stomodaea appeared in solutions that had previously been inhibitory.

3. *Exposure of newly fertilized eggs to zinc chloride for six and twenty-four hours*

Eggs treated with *M*/10,000–*M*/40,000 zinc chloride solutions for only the first six hours developed into inhibited, bilateral plutei when returned to sea water. At 48 hours these embryos possessed short anal arms at aberrant angles, poorly differentiated oral lobes, and 10 per cent exogastrulation. Such inhibition was more noticeable in the higher concentrations. Similar treatment with *M*/80,000–*M*/320,000 showed little or no effects of the agent.

When newly fertilized eggs were treated for the first 24 hours with *M*/10,000–*M*/20,000 zinc chloride and then returned to sea water they developed chiefly (by 48 hours) into radial forms with differentiated entera and basal rings of cilia (Figs. 18–22). At least 50 per cent of these embryos showed considerable polar elongation (Figs. 21, 22). In the weaker solution, polar elongation was more pronounced and stomodaea were commonly present. With 24 hours exposure to intermediate concentrations (*M*/40,000–*M*/80,000) all of the larvae developed radial symmetry, well-differentiated entera (with 10 per cent exogastrulation) but no skeleton (Figs. 23–30). Approximately 50 per cent showed apical lobes or extensions (Figs. 28–30) which were often quite large. Up to 50 per cent showed the mouth at the extreme apical end (Fig. 24) while many showed the mouth at one side of an apical lobe (Figs. 25–27).

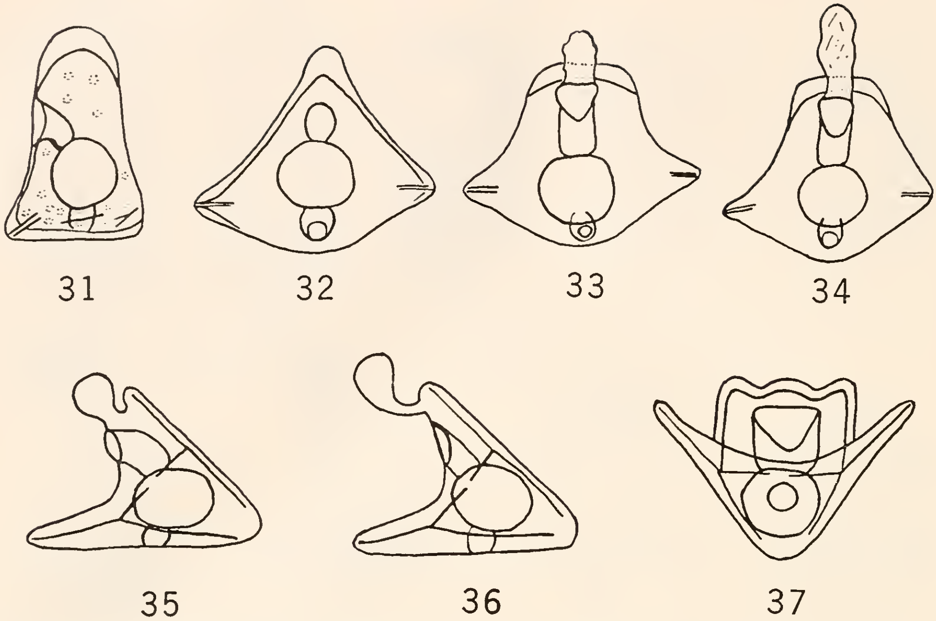
Following 24 hours treatment with low concentrations (*M*/160,000–*M*/320,000) the eggs developed chiefly into bilateral plutei showing all degrees of inhibition (Figs. 31–37). Anal arms were short and at increased angles. Modifications were more pronounced in the stronger of these two solutions (Figs. 31–34) where a few approached the elongated radial forms (Fig. 31). Following exposure to the weaker solution (*M*/320,000) a few developed into plutei that were not far from the controls (Fig. 5) except for broad, poorly-differentiated oral lobes and slightly shortened anal arms at increased angles (Fig. 37). All developed some skeleton. One of the most interesting modifications found in these forms was the appearance of a thin-walled sac in the region of the upper lip of the stomodaeum (Figs. 33–36). This sac was undoubtedly a small apical lobe, comparable in origin to the apical lobes of more modified embryos (Figs. 25–27) and arising from a



FIGURES 18–30. Figures 18–22, 48-hour larvae treated for the first 24 hours with *M*/10,000–*M*/20,000 zinc chloride and then returned to sea water. Figures 23–30, 48-hour larvae treated for the first 24 hours with *M*/40,000–*M*/80,000 zinc chloride and then returned to sea water.

region that had escaped the controlling factors normally found in the differentiation of an oral lobe.

In general, these experiments have demonstrated that high concentrations of zinc chloride give over-all inhibition if administered only during cleavage (first six hours) while low concentrations have little or no effect if used only during cleavage. It was also shown that the removal of larvae from zinc chloride solutions after 24 hours permits a greater degree of differentiation than when the ex-

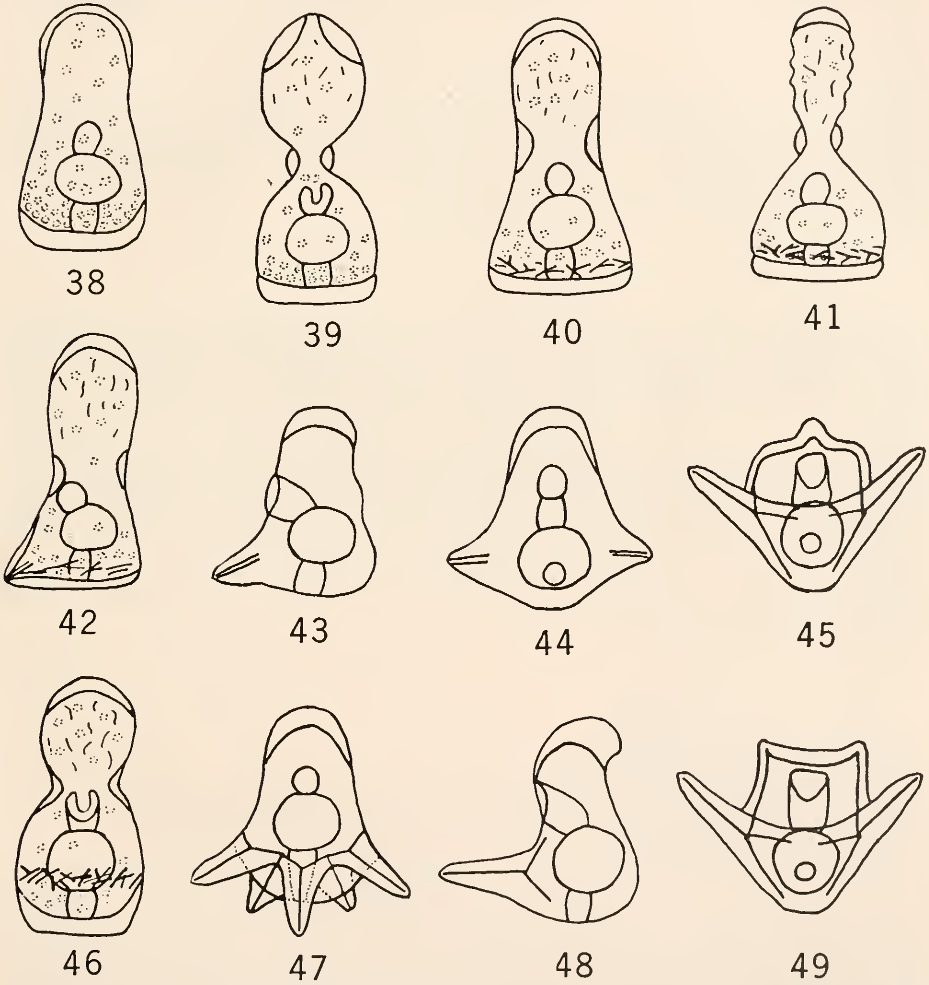


FIGURES 31–37. 48-hour larvae treated for the first 24 hours with $M/160{,}000$–$M/320{,}000$ zinc chloride and then returned to sea water.

posure was continuous. In high ($M/10{,}000$–$M/20{,}000$) and intermediate ($M/40{,}000$–$M/80{,}000$) concentrations for 24 hours there was radial symmetry and polar elongation but with apical and basal lobes developing and differentiating. Stomodaea also appeared in these experiments which may indicate that the factors responsible for such differentiation are not very sensitive to inhibition during the first 24 hours. The appearance of the stomodaeum at the apical end may indicate a part of the phenomenon of ventralization in which the ventral area extends more rapidly than the dorsal and in this case pushes the stomodeal region apically.

4. *Exposure of mid-blastulae to zinc chloride for six and eighteen hours*

Whereas six hours exposure to high concentrations of zinc chloride, beginning immediately after fertilization, gave only over-all inhibition and lower concentrations had little or no effect the situation was quite different when the 6-hour blastula was exposed for a similar period. Six hours exposure to $M/10{,}000$–$M/20{,}000$ gave 75–90 per cent radial elongated forms (Figs. 38–41) with thin apical regions or lobes. The remainder graded into inhibited bilateral plutei (Figs. 42–45). Some of these were radial and enlarged apically but bilateral basally (Figs. 42, 43). In $M/20{,}000$, most of the bilaterals had skeleton and most of the elongated radials had rings of skeletal spicules around their bases (Figs. 40, 41). No exogastrulae were found. Approximately 60 per cent of the blastulae exposed for six hours to $M/40{,}000$ developed into bilateral forms (as Figs. 42–45), commonly elongated in the polar direction (Figs. 42–44) but with short anal arms. The remaining 40 per cent were radial and usually elongated. All of these had circles of skeletal spicules around their bases and many formed circles of short arms (Figs. 46, 47). Following treatment with *M*/80,000 most of the larvae developed bilaterality and two anal arms at increased angles (Figs. 48, 49). The oral lobes were commonly extended and poorly differentiated (Fig. 48). There were many irregularities and an occasional exogastrula. Treatment with *M*/160,000–*M*/320,000 gave chiefly normal plutei although there were still a few minor modifications to be found in the stronger of these two solutions.



38 39 40 41

42 43 44 45

46 47 48 49

FIGURES 38–49. Figures 38–41, 48-hour larvae exposed for six hours to *M*/10,000–*M*/20,000 zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water. Figures 42–47, 48-hour larvae exposed for six hours to *M*/40,000 zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water. Figures 48–49, 48-hour larvae exposed for 6 hours to *M*/80,000 zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water.

The exposure of blastulae to the various concentrations of zinc chloride for 18 hours, and followed by return to sea water, gave forms that were very similar to those from exposure to the same solutions for 24 hours beginning shortly after fertilization. Skeleton and slight bilaterality was found in a few at *M*/80,000. Exogastrulae occurred only occasionally but stomodaea opened at the apical end (Fig. 24) in as high as 50 per cent following treatment with *M*/40,000–*M*/80,000.

These experiments show that six hours exposure to zinc chloride after cleavage is highly effective in causing the development of radial symmetry and polar elongation. This is to be contrasted with exposure during the six-hour period of cleavage which is relatively ineffective. Skeleton was not greatly inhibited during the second six hours of treatment and commonly differentiated with reference to a radial symmetry even to the extent of appearing in a circle of radial arms. That treatment during the cleavage period had some importance, however, was demonstrated by exposure to *M*/80,000 for 18 hours following cleavage. Here skeleton and slight bilaterality appeared in a few larvae as contrasted to their complete absence when treatment began immediately after fertilization.

5. *The effects of zinc chloride when administered with glutathione*

Since zinc is known to react with the sulfhydryl groups of certain enzymes it seemed advisable to determine any effects on morphogenesis caused by the addition of a sulfhydryl compound to the experimental solutions of zinc chloride. The present work is preliminary and incomplete since only one sulfhydryl compound (glutathione) was used and all possible experimental variations were not investigated. One questionable procedure in these experiments was the addition of glutathione to sea water (which possibly led to its partial oxidation) but since the eggs of *Dendraster* develop in a sea water medium there seemed to be no other alternative.

Newly fertilized eggs, and in some cases six-hour blastulae, were placed in the following sea water solutions:

1. Sea water (control)
2. 0.1 per cent glutathione
3. 0.05 per cent glutathione
4. O.025 per cent glutathione
5. *M*/40,000 zinc chloride
6. 50 cc. *M*/20,000 zinc chloride plus 50 cc. 0.05 per cent glutathione
7. *M*/80,000 zinc chloride
8. 50 cc. *M*/40,000 zinc chloride plus 50 cc. 0.05 per cent glutathione

It was found that a sea water solution of 0.1 per cent glutathione (sol. 2) did not permit cleavage beyond the two-cell stage and commonly prevented it altogether. With solution 3 development occurred and stunted plutei differentiated (as Fig. 14). Of these, approximately five per cent had evaginated entoderms. Blastulae placed in solution 3 also gave inhibited plutei. Eggs and blastulae exposed continuously to solution 4 (0.025 per cent glutathione) differentiated into plutei that were very close to the control (Fig. 5). A few, however, showed slight inhibition of the oral lobe.

Eggs exposed to solution 5 ($M/40{,}000$ zinc chloride) developed, as previously described, into radial larvae, with basal ciliated bands (Figs. 8–11). Of these, approximately 50 per cent had apical extensions and 10 per cent had exogastrulated. In solution 6, normal plutei, that could not be distinguished from the control, developed even though the concentration of zinc was identical with that of solution 5. Similar results were obtained with solutions 7 and 8 and in experiments starting with the blastula as well as with the one-cell stage. These experiments were repeated and in every case the concentration of glutathione tested gave complete protection from the above concentrations of zinc.

Discussion

These experiments on the modification of developmental patterns in the sand dollar with zinc chloride have shown several interesting things. The cleavage period (first six hours) was not particularly susceptible to this agent except in the higher concentrations. When inhibition did occur it was general. That is, the embryos were inhibited proportionately and developed into stunted bilateral plutei with short anal arms and poorly developed oral lobes. It would seem from these observations that certain processes (enzymatic and otherwise) that are concerned with later differentiations are not very active during the cleavage stage and are therefore less susceptible to zinc. This is in line with the work of Gustafson and Hasselberg (1951) which showed that in the sea urchin embryo the activity of a number of enzymes was constant up to the mesenchyme blastula stage and then increased.

That treatment during cleavage to concentrations of zinc, which do not affect rate, does have some effect on morphogenesis is shown by a comparison of the different experiments. When exposure was begun immediately after fertilization and continued for 24 to 48 hours the embryos showed a higher percentage of exogastrulae and failed to develop stomodaea and skeleton as readily as those in which exposure to zinc was begun after cleavage was completed. Such experiments show that while the larva may recover from the effects of lower concentrations when administered only during the cleavage stage, the inhibition may persist and enhance the effects of exposure after cleavage.

The second six-hour period of development is considerably more sensitive to zinc chloride than is the first six-hour period. Also, at this stage the effects are differential; that is, some processes are inhibited greatly while others are inhibited little if any. This is particularly shown in the development of radial symmetry and in polar elongation. At first glance it would appear that radial symmetry is due to the inhibition of bilaterality but this does not seem to be the case. Rather it appears to be a phenomenon of ventralization (see Rulon, 1949) brought about by the inhibition of dorsal and the spread of ventral regions. In normal development, Child (1941) has shown a gradient in the indophenol oxidase (cytochrome oxidase) reaction which is high at the ventral side but low at the dorsal. It would seem that zinc chloride has less effect on cytochrome oxidase activity than on other activity (perhaps largely enzymatic) located toward the dorsal side. As a consequence the ventral area spreads until it encircles the embryo. Evidence for this is in the radial plutei, which are completely ventralized, with gradations through plutei with unusually large ventral areas and are only partially ventralized.

Polar elongation also seems to be related to gradient differences but in this case those extending from apical to basal regions. Here, again, Child (1941) has shown a differential in indophenol oxidase with the highest activity being at the animal pole. If it is assumed that zinc chloride has less inhibitory effect on enzymatic activity near the apical than near the basal area it may easily be seen how the apical region grows at the expense of basal regions and the embryo elongates in the polar direction. With such elongation the correlating influences between the two regions become farther and farther removed from each other and differentiation of the separated regions becomes more and more independent. In weak solutions, and in embryos removed to sea water, conditioning and recovery phenomena become more apparent with the formation of differentiated apical lobes, apical tufts, stomodaea, etc.

The general effects of zinc chloride are very similar to those of sodium selenite (Rulon, 1952) and cobaltous chloride (Rulon, 1953a) although zinc is effective in approximately one-tenth the concentration required by cobalt or selenium. The effects of these three agents are very different from those of such agents as sodium azide (Rulon, 1950) which strongly inhibit the activity of cytochrome oxidase. Agents which do inhibit this important respiratory enzyme also profoundly inhibit apical development, cause a decrease in size of the ventral area and commonly induce evagination with progressive entodermization.

Zinc, cobalt and selenium have been shown to be inhibitors of sulfhydryl (Potter and Dubois, 1943; Marston, 1952, and others)—in fact, this seems to be one of the few things these ions have in common. A number of enzymes (including succinic dehydrogenase) seems to require the sulfhydryl radical for action (Sumner and Somers, 1953). Cytochrome oxidase does not. In the present work experiments were conducted which showed that eggs and blastulae would develop into normal plutei in solutions of $M/40{,}000$ and $M/80{,}000$ zinc chloride if the solutions also had a concentration of 0.025 per cent glutathione. Whether this protection was through reaction of glutathione with zinc inside the cell, at the cell surface, or in the solution outside the cell was not determined. These experiments did not show that zinc was effective in modifying morphogenetic pattern by reacting only with thiol groups in the cell but they may indicate that such a reaction was likely and that at least part of the effect of zinc in these experiments may have been through the inhibition of sulfhydryl radicals. Preliminary experiments (unpublished) have shown that the eggs and young larvae of the sand dollar may similarly be protected from the effects of cobalt and selenium by the addition of glutathione to the test solutions.

SUMMARY

1. Newly fertilized eggs and 6-hour blastulae were exposed to sea water solutions of zinc chloride ($M/10{,}000$–$M/320{,}000$) for varying intervals of time.

2. It was found that high concentrations inhibited cleavage while low concentrations caused marked changes in the developmental pattern without much effect in cleavage. The continuous exposure of newly fertilized eggs to strong solutions following fertilization resulted in radial, poorly-differentiated larvae but as the strength of the solution decreased the larvae became elongated in the polar direction and differentiated basal ciliated bands. In the weaker solutions, plutei with large

ventral areas differentiated. In $M/10,000$, exogastrulation occurred in 10–25 per cent of the larvae while the differentiation of skeleton was prevented in all concentrations above $M/80,000$.

3. Continuous treatment after cleavage gave larvae that were but slightly different from those treated continuously from fertilization. The chief differences were that fewer exogastrulae developed, skeleton differentiated in slightly higher concentrations, and stomodaea developed in solutions that had previously been inhibitory(when treatment began with the one-cell stage).

4. Exposure to high and intermediate concentrations for the first 24 hours following fertilization (followed by return to sea water) caused the larvae to show radial symmetry, polar elongation, and the differentiation of apical and basal lobes. Six hours exposure immediately after cleavage was highly effective in causing the development of radial symmetry and polar elongation, as contrasted to exposure during the six-hour period of cleavage which was relatively ineffective.

5. Developing eggs may be protected from the effects of zinc chloride by adding glutathione to the test solutions. Since the effects of zinc are very similar to those of cobalt and selenium it is suggested that part of the effect of zinc in these experiments may be through the inhibition of enzymes with sulfhydryl radicals.

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MICRO GASOMETRIC DETERMINATION OF DISSOLVED OXYGEN AND NITROGEN[1]

P. F. SCHOLANDER, L. VAN DAM, C. LLOYD CLAFF AND J. W. KANWISHER

Woods Hole Oceanographic Institution, Marine Biological Laboratory, Woods Hole, Massachusetts, and Harvard Univ. Medical School, Boston, Massachusetts

For polluted water and many biological fluids the Winkler method for the determination of dissolved oxygen may easily become unreliable, or inapplicable. This difficulty can be avoided by using gasometric methods. We describe below such a method for the determination of oxygen (and nitrogen) in one cubic centimeter of water. It has been used extensively under field conditions to analyze, for instance, mud water in mangrove swamps (Scholander, Van Dam and Scholander, 1955), the sap in grape vines (Scholander, Love and Kanwisher, 1955), and the gases in icebergs (Scholander, Kanwisher and Nutt, 1955). The principle and apparatus are based on a micro method for the determination of carbon monoxide in blood (Scholander and Roughton, 1942).

PRINCIPLE

The water sample, followed by acid, is drawn anaerobically into an extraction syringe pipette, containing bicarbonate in the dead space. The dissolved gases escape into the large CO_2 gas phase generated by the reagents. The liquids are ejected, and the CO_2 is absorbed by strong KOH, leaving the extracted oxygen and nitrogen as a bubble which is moved into the capillary of the extractor. The gas bubble is transferred to a syringe analyzer, where the oxygen is absorbed.

APPARATUS

The apparatus consists of two parts: (1) combined water sampler and gas extractor, and (2) gas analyzer.

The extractor (Fig. 1B) consists of a 5-cc. syringe, with a fused capillary tip 5 cm. long, and of 0.7 mm. bore. It is provided with a releasable spring drag, made by passing a wire through a rubber cuff. The syringe is mounted on a wood or plastic base provided with a levelling screw, so that the tip of the capillary can be set to match the cup on the gas analyzer. The capillary tip can be closed with a rubber stopper which has a small hole slit at one end, and can also be provided with a glass cup for KOH (Fig. 1, D and E).

The gas analyzer (Fig. 1A) consists of a millimeter-graduated 0.6-mm. precision bore capillary, fused to a $^{3}/_{16}$-inch precision bore glass tubing, which acts as a barrel for a stainless steel plunger, terminating in an $^{8}/_{40}$ screw. The screw is engaged by a spring-loaded lever arm. The analyzer is temperature-stabilized by a plastic water jacket, fluted along the edges and resting on legs to keep it insulated from direct contact with hands and table.

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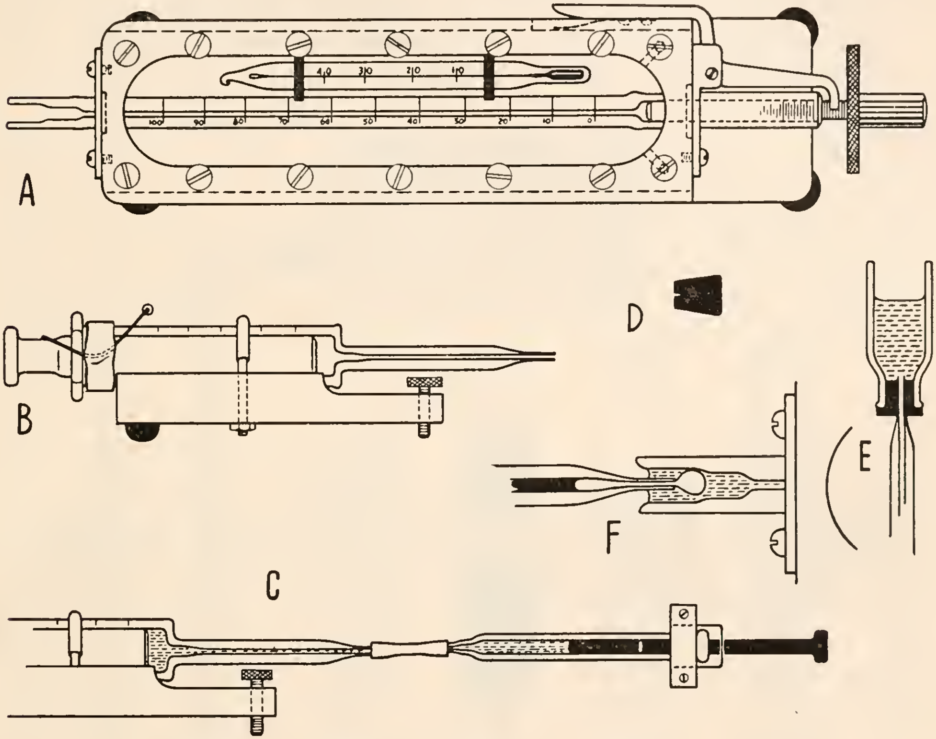

FIG. I

Figure 1. A, gas analyzer. B, extractor. C, transfer of water sample from syringe burette to extractor. D, rubber stopper for closing of extractor. E, KOH cup placed on extractor tip. F, transfer of extracted bubble to cup of gas analyzer.

Reagents

A. For extracting gases from sample

1. Carbonate solution: Dissolve 8 g. sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$ and 5 g. K_2CO_3 in 20 cc. water.
2. Acid solution: Dissolve 10 g. citric acid and 3 g. sodium citrate in 20 cc. water.
3. Strong KOH solution: Add 100 g. KOH to 60 cc. water in a beaker.

Store (1) and (2) in Erlenmeyer flasks.

B. For gas analysis

1. Acid citrate: Dissolve 170 g. sodium citrate and 6 g. citric acid in 200 cc. water.
2. Alkaline citrate: Dissolve 70 g. sodium citrate and 5 g. KOH in 120 cc. water.
3. Oxygen absorber: Add 15 g. pyrogallol to 100 cc. 20% NaOH in a rubber-stoppered bottle and cover with a layer of paraffin oil 2 cm. thick. The pyrogallol is dissolved under the oil by stirring with a glass rod.
4. Acid rinsing solution: Add 1 cc. concentrated sulfuric acid to 500 cc. water and add 10 mg. potassium permanganate.

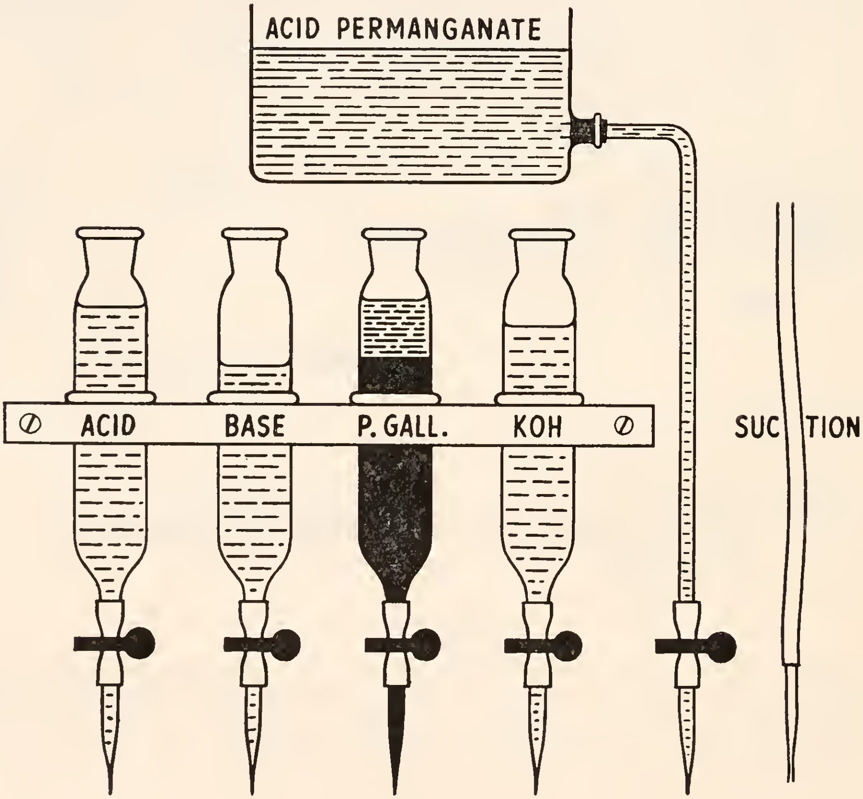

FIG. II

Figure 2. Rack for reagents and suction.

The reagents are stored as shown in Figure 2. Solutions A1 and A2 are highly concentrated in order to keep the gas solubility low. The salts crystallize out when cold, so it is often necessary to redissolve them by heating. The solutions are boiled when made up, to overcome the gas supersaturation that results from dissolving the salts.

Calibration

The capillary of the gas analyzer is most easily calibrated by means of a micrometer burette (Scholander, 1942a), with the tip above the bulb running horizontally. With the analyzer mounted horizontally on a stand, and the screw plunger removed, a stiff rubber connection is made between the water-filled cup of the capillary and the tip of the burette, being careful not to trap any air bubbles in the connection. The water is *slowly* and *evenly* moved back in the capillary by means of the micrometer, reading off partial as well as total calibration.

For accurate work it is best to draw the sample into an accurately calibrated syringe burette, with or without automatic stop, as in Figure 1C (Scholander, 1947), and from there deliver it into the extractor syringe. In many cases it will, however, suffice to measure the water sample directly into the extractor, which has been calibrated by delivering water from the one-cc. mark until the plunger touches bottom. During this operation the number on the plunger is kept facing up. The water is delivered into a cylindrical shell vial, about 1 × 3 cm., silicon-coated on the inside. The vial is handled only by forceps. The tall sides and absence of thermal convection effectively prevent evaporation loss during weighing (Scholander, Edwards and Irving, 1943). For repeated deliveries remove the water by means of suction.

Procedure

Collecting sample and extraction of gas

Pour the boiled acid and alkaline solutions (A1 and A2) into two 10-cc. beakers, which are kept covered. Rinse the extractor with water. Draw in ½ cc. of carbonate solution, discard this, and fill the dead space with the same, orienting the plunger with the number up. Either draw in the one-cc. water sample directly or push it in from the burette (Fig. 1C) through a short piece of rubber or plastic tubing. A long and thin-walled polyethylene tube is seriously pervious to gases and should not be used. Trapping of air bubbles must, of course, be scrupulously avoided. The extractor is now held vertically with the tip in the acid, and 0.2 cc. is carefully drawn in. This layers under the carbonate solution, and there is time to close the capillary tip with the rubber stopper before the CO_2 evolution begins. The loss of a few cubic millimeters of gas or liquid at this point is immaterial. With the plunger clip released, the extractor is shaken until CO_2 evolution stops, which with the proper strength of carbonate solution (A1) should be at a gas phase of about 4 cc. Shaking should be continued for two minutes, which is conveniently done by placing the extractor on a rotator. Holding the extractor tip down, the plunger is forced in to give a slight over pressure. The stopper is removed and the extracted water is allowed to run down into a finger bowl of water. Keeping the tip under water as a safety measure all of the syringe fluid is disposed of, leaving only a seal in the capillary. Turn the extractor tip up, readjusting the plunger so as to maintain the liquid seal in the capillary, and attach the KOH cup (Fig. 1E). Fill this half way up with strong KOH (A3). Expel into the KOH any air trapped in the capillary. Draw down the KOH over the CO_2 in the barrel, letting the plunger in as the CO_2 is absorbed. At the very last stage of the absorption, run the remaining bubble of oxygen and nitrogen up into the capillary, where it is safely protected from diffusion exchange until it is transferred to the analyzer.

Transfer and analysis of gas bubble

The analyzer is rinsed and the dead space plus the cup is filled with the alkaline citrate solution (B2). With the extractor and analyzer placed on the table, the extractor tip is introduced into the citrate which fills the cup of the analyzer, and the gas bubble is carefully transferred into the cup by gently twisting the extractor plunger in (Fig. 1F). The analyzer is held cup down, and the gas bubble is drawn into the capillary. With the cup up, the bubble is drawn down into the barrel, and then brought back very *slowly and evenly* into the capillary for a reading. Any abrupt movement of the meniscus leaves excess liquid on the sides of the capillary, and the volume of the bubble will be estimated too large. Enough of the citrate above the gas bubble is drawn off so that both menisci of the seal remain within the capillary during the reading. If the upper meniscus is in the cup, the gas volume reads significantly too large. The readings are made with a hand lens and with the analyzer resting on the table. The length of the bubble is checked by repeating the procedure, and the smallest reading is used (V_1). Oxygen is absorbed by slowly and steadily drawing one cupful of pyrogallol down over the bubble, with the instrument held cup up. The pyrogallol layers around the bubble in the barrel on top of the citrate. By this procedure fresh pyrogallol, in excess, runs over the bubble, which effectively prevents the formation of monoxide. The remaining nitrogen [2] bubble is slowly moved up into the capillary, and the volume is read with all three menisci in the capillary (V_2). Read the temperature of the analyzer.

After analysis, rinse the analyzer several times in water under the faucet, disconnecting the screw feed and removing the plunger. If the water supply is limited, rinsing with permanganate solution (B4) until this retains its color may be necessary.

CALCULATIONS

The oxygen contained in the sample equals $(V_1 - V_2 - C)$ $(F_s \times i) \times F_a$, where C is the blank correction for oxygen in the reagents, F_s is the factor reducing measured gas volumes to standard conditions (STPD), i is the factor for gas remaining in the liquid phase (near to 1.0075 when liquid volume/gas volume is ¼), and F_a is the calibration of the analyzer capillary. F_s is based on (1) a temperature corrected barometer reading, from which is subtracted the water vapor tension at the analyzer temperature, and (2) the temperature of the analyzer (Handbook of Chemistry and Physics, 1952, pp. 2130 ff. and 1910 ff.).

The nitrogen contained in the sample equals $(V_2 - C)$ $(F_s \times i) \times F_a$, where C is the blank correction for nitrogen in the reagents, F_s is the same as above, i is near to 1.004, and F_a is the same as above.

When the analysis is performed at a temperature between 15° and 30° C. the following empirical terms can be used, with less than 0.2% error, as substitutes for $(F_s \times i)$, namely, for oxygen $F_s \times i = 0.215\ B/t + 160$, and for nitrogen $F_s \times i = 0.214\ B/t + 160$, where B is the uncorrected barometric pressure in mm. Hg and t is the temperature of the analyzer in centigrade degrees.

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[2] "Nitrogen" here includes the noble gases and other non-absorbable components, like methane, if such are present.

DETERMINATION OF REAGENT BLANK C

Half a liter of water is boiled in an Erlenmeyer flask, provided with a cork through which a capillary glass siphon fits loosely. The free end of the siphon has a short piece of rubber tubing with a pinchcock. After half an hour of steady boiling, 10 cc. of the still boiling water are transferred anaerobically through the siphon to a 10-cc. syringe. This is closed with a fine bore rubber tube and glass plug and is cooled under the faucet. The gas-free water is quickly transferred from this into the extractor by means of a short and heavy-walled tubing. Air bubbles must be scrupulously avoided. The extraction and analysis proceed as described. Our blanks amount to 0.8 mm. for oxygen and 2.4 mm. for nitrogen.

CHECKS ON AIR-EQUILIBRATED WATER

Fifty cc. distilled water are rotated in a 500-cc. open bottle which is kept partly submerged in a water bath of room temperature. Bubbling of air through water results in an unknown degree of supersaturation and cannot be used in this connection. After a half hour's equilibration samples may be taken out. The dissolved oxygen in the sample equals

$$\frac{\alpha \times (B - b - w)}{760} \times 0.2094,$$

where α is the solubility coefficient, B is the read barometric pressure, b the temperature correction for the barometric reading, w the water vapor tension at the water bath temperature, and 0.2094 the oxygen fraction in the air.

ACCURACY

The method has been tested on air-equilibrated water as described above. Out of 21 consecutive oxygen analyses 16 were within ± 1% of the theoretical value, 4 were within ± 2%. The average for 20 determinations was 100.1%.

In the same series, 17 nitrogen determinations were within ± 1% of the value given in the Handbook of Chemistry and Physics (1952), and 3 were within + 2%. The average of 20 determinations was 100.6%.

USE OF THE INSTRUMENT FOR STRAIGHT GAS ANALYSIS

For simple gas analysis the analyzer is charged with the acid citrate solution (B1), and a gas bubble is deposited in the cup, using a syringe technique as described by Scholander, Claff, Teng and Walters (1951), or a mercury pipette (Scholander, 1942b). Thanks to the smooth screw control and the water jacket, an accuracy of ± 0.2% or better can easily be obtained for CO_2, O_2, and nitrogen. At high carbon dioxide concentrations (20% or more) a slight loss of CO_2 must be expected, unless the sample is drawn into a mercury pipette and delivered into mercury which fills the analyzer cup (cp. Scholander, 1942b).

We are much indebted to Mr. J. D. Graham, Department of Physiology, University of Pennsylvania, for suggesting the precision bore tubing for the barrel and for much advice in the development of the apparatus. We also wish to acknowledge the craftsmanship of Mr. J. L. Allen at the instrument shop of the Woods Hole Oceanographic Institution, who built the first models, and of Image Transfer Inc. of Randolph, Massachusetts, which engineered the final models.

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ABSTRACTS OF SEMINAR PAPERS

July 5, 1955

The role of divalent cations in chromosome structure and crossing over. R. P. Levine.

The mechanism by which genes recombine through crossing over remains among the most poorly understood genetic phenomena. It is clear, however, that crossing over is intimately related to chromosome structure. Evidence has been accumulating suggesting that divalent cations may play a role in chromosome structure. Steffensen has demonstrated that spontaneous chromosome breakage in Tradescantia is increased seventeen times for plants raised in a calcium-deficient medium. Mazia has shown that salivary gland chromosomes of *Drosophila melanogaster* can be dissociated *in vitro* by treatment with a chelating agent ("Versene"). The work reported here is concerned with the effect of altered ionic conditions on crossing over in *D. melanogaster.* Adult females (heterozygous for the X-chromosome markers *yellow* body color, *cut* wings, *raspberry* eye color, and *forked* bristles) when fed on the usual medium supplemented with 0.1 *M* calcium chloride display a significantly reduced per cent crossing over from the sixth to fourteenth day after feeding. On the other hand, if larvae are fed on the usual medium which has been made 0.01 *M* for "Versene," significantly increased crossing over is observed. If crossing over results from altering ionic conditions within or in the environment of the chromosomes, then it would be predicted that the amount of crossing over or chromosomal stability would be related to the amount of divalent cation present. The data presented here are in accord with such a prediction. Thus, the feeding of excess calcium has resulted in significantly reduced crossing over, while an ion deficiency, such as might be produced by a chelating agent in the medium, results in significantly increased crossing over. The data presented here suggest, therefore, that crossing over can be modified by ionic changes according to predictions as to their effects on chromosome structure.

The distribution of parental material in chromosome reproduction. Daniel Mazia and Walter S. Plaut.

When a chromosome reproduces itself, the total amount of chromosomal material is doubled and is divided equally between the daughters. One may imagine: (1) that the parent chromosome is producing a replica of itself out of new material or (2) that the material of the parent chromosome is equally distributed between the daughters. The following experiment was designed to test these alternatives.

Desoxyribonucleic acid (DNA) was selected as being representative of chromosome material. Seedling roots of *Crepis capillaris* (diploid chromosome number = 6) were incubated for 12 hours in a medium containing C^{14}-labelled thymidine, a precursor to DNA. The material was fixed (removing the unincorporated thymidine), and squash preparations were covered with stripping film for high resolution autoradiography. The amount of C^{14} incorporated into individual nuclei or groups of chromosomes was assessed by a modification of the method of Mazia, Plaut and Ellis (1955).

Late anaphases and telophases were measured, since at these stages it is possible to identify sister groups of chromosomes unequivocally. The presence of radioactive DNA indicated that the last 12 hours of interphase, in *Crepis,* includes at least part of the period during which DNA is being synthesized.

If the parental DNA (and hence the newly formed DNA) were equally distributed between the daughter chromosomes, then sister nuclei would ncessarily be equally labelled. If parent chromosomes form new chromosomes, there should be 6 radioactive chromosomes and 6 non-radioactive (or less radioactive) chromosomes. When sister chromosomes separate randomly at anaphase, there should be a certain proportion of cases where sister nuclei receive unequal numbers of labelled chromosomes and therefore show unequal radioactivity.

The measurements have revealed cases of the distribution of the labelled DNA with varying degrees of *inequality* as well as cases of near-equal distribution. The extent of inequality that may be observed is such that it is difficult to attribute it to experimental variability. Therefore, it is concluded that the parental chromosome material is *not* equally distributed between the daughters, as would be called for by certain current theories. Whether the daughter chromosome is entirely new or receives a limited amount of material from the parent cannot be decided by the data thus far obtained.

The lethal radiation effects of x-rays and fast neutrons on the embryo of the American cockroach. MAX A. LAUFFER AND HERMAN CEMBER.

Embryos of the cockroach, *Periplaneta americana,* at an average age of 14 days, were irradiated with 50 and 100 kV filtered x-rays, having effective wave-lengths of .479 and .563 Å, and also with fast neutrons of energies between 2 and 10 million electron volts. X-ray doses varied between 25 and 5,000 roentgens and dose rates between 25 and 100 roentgens per minute. Neutron doses varied betwen 41 and 4426 rep. After irradiation, the embryos were incubated at 27° C. for 70 days. At the end of that time, the fraction of nymphs which hatched completely was determined and was compared with the fraction in unirradiated controls. Details of these experiments are described in Cember's thesis on file at the University of Pittsburgh.

The fractions surviving various doses of x-irradiation fit the target theory for the case of one hit each on three targets, with a dose, D_0, corresponding to an average of one hit per target, of 1090 roentgens. They also fit a single target two-hit theory with the value of D_0 of 1000 roentgens. The effect was independent of wave-length and of dose rate. The results of the neutron-irradiation experiments fit a single target single-hit theory with a D_0 of 170 rep.

It is impossible to rationalize the discrepancy between the D_0 values for x-irradiation and for neutron-irradiation in terms of a simple direct-hit target theory, even by assuming that the targets are in the form of long filaments. The targets would have to be impossibly thin, compared to atoms, to account for the observations. The results can be understood, at least qualitatively, in terms of the modified target theory discussed by Zirkle.

JULY 12, 1955

Differentiation of mixed aggregates of dissociated tissue cells. J. P. TRINKAUS AND PEGGY W. GROVES. No abstract submitted.

Chromosome movements and cell division after spindle destruction by irradiation of cytoplasm. R. E. ZIRKLE, W. BLOOM AND R. B. URETZ. No abstract submitted.

Changes in refractive index of irradiated chromosome segments. R. B. URETZ, W. BLOOM AND R. E. ZIRKLE. No abstract submitted.

JULY 19, 1955

Purification and properties of an enzyme system which reversibly cleaves succinate to form two molecules of acetyl coenzyme A. GERALD R. SEAMAN.

An enzyme system which catalyzes the cleavage of succinate has been obtained from the ciliated protozoan, *Tetrahymena pyriformis.* Purification steps involving freezing and thawing, ammonium sulfate fractionation, acetone precipitation, and adsorption and elution from calcium phosphate gel, result in a purification of the enzyme of approximately 300-fold. Stoichiometry of the reaction catalyzed by the purified system indicates that the cleavage system may be formulated as:

$$\tfrac{1}{2}\ \text{succinate} + \tfrac{1}{2}\ \text{DPNH} + \text{Co A} + \text{ATP} + \tfrac{1}{2}\ \text{H}^{+} \rightleftarrows \text{acetyl Co A} + \tfrac{1}{2}\ \text{DPN}^{+} + \text{ADP} + \text{Pi}$$

A naturally occurring inhibitor for alkaline ribonuclease. JAY S. ROTH.

An inhibitor, active against crystalline pancreatic ribonuclease (PRNase) and alkaline RNase of kidney and liver mitochondria, occurs in the supernatant fraction of rat tissues. These fractions were prepared by centrifuging 1 : 10 water or 0.25 *M* sucrose homogenates at 60,000 g for 1½ hours. The inhibitor, which is non-dialyzable and is destroyed by heating at 65° for 5 minutes, is probably a protein and may be identical with a lipoprotein-heparin complex described by Snellman and Sylvén. An assay system for the inhibitor was devised, with one unit defined as the amount of inhibitor that gives 50% inhibition of the activity of 0.015 γ of PRNase under standard conditions. Using this assay system the inhibitor was found to be present in brain, liver, lung, spleen, heart, kidney and muscle in decreasing amounts. Liver inhibitor, although capable of inactivating between 0.25 and 0.50 γ of PRNase per ml. of supernatant, already has bound to it large quantities of intracellular RNase. Quantitatively the bound RNase is somewhat larger in amount than the total RNase activity of mitochondria measured at pH 7.8. The bound RNase is released and the inhibitor inactivated by certain SH reagents. p-Chloromercuribenzoic acid and Pb^{++} at 4×10^{-4} *M* concentration are equally effective, while p-iodosobenzoic acid is less so and iodoacetamide and iodoacetic acid have no action. Heating the supernatant fraction (65° for 5 minutes) destroys inhibitor activity but it does not destroy the activity of the bound RNase which may be released by SH reagents. Contact with 0.25 *M* H_2SO_4 at 0° for 24 hours also destroys inhibitor activity which explains the large increase in RNase activity when tissues are treated in this manner. RNase inhibitor may act as an intracellular regulator of RNase activity. (Aided by grants from the National Institutes of Health and the Damon Runyon Memorial Fund.)

Recent aspects of the metabolism of uridinediphospho-glycosyl compounds. E. P. ANDERSON AND H. M. KALCKAR.

Uridine triphosphate (UTP) has been shown to play a specific role in the synthesis of glucuronides, since uridinediphospho-glucose (UDPG), formed by reaction of the nucleotide with α-glucose-1-phosphate (G-1-P), (UTP + G-1-P $\rightleftarrows$ UDPG + PP), can be directly oxidized to uridinediphosphoglucuronic acid (UDPGA). When nucleoside triphosphates were incubated with G-1-P in the presence of UDPG pyrophosphorylase and -dehydrogenase, and subsequently a phenol acceptor and liver microsomes (coupling enzyme) added, the phenolglucuronide formed in the reaction with UTP was seven-fold greater than that formed with adenosine triphosphate (ATP). Since the oxidation of UDPG as well as of G-1-P can be followed spectrophotometrically in the presence of the proper enzymes and pyridine nucleotide, these reactions permit sensitive and specific analyses for the uridine nucleotide compounds. This series of reactions from G-1-P also demonstrates the link between glycogen phosphorolysis and glucuronide formation. If the system is further linked with hexokinase and ATP, free glucose can serve as glycosyl source. It has therefore been possible to obtain UDPG labeled with C^{14} in the glucose moiety from UTP and glucose-C^{14} (labeled uniformly or specifically in carbon atom 6) by incubation in a system containing hexokinase, ATP, phosphopyruvate, pyruvate phosphokinase, phosphoglucomutase, UDPG pyrophosphorylase, and inorganic pyrophosphatase. The glucose-C^{14} was found to be incorporated without isotopic dilution and the system gave yields up to 80–95% of UDPG from glucose.

Some recent experiments on electrokinetic separation of proteins and of microorganisms. ALEXANDER KOLIN. No abstract submitted.

JULY 26, 1955

Tactic responses in purple bacteria. RODERICK K. CLAYTON.

Rhodospirillum rubrum displays a negative phototaxis, a positive aerotaxis in the dark and in dim light, and a negative aerotaxis in bright light. At a moderate light intensity the bacteria congregate in a zone of intermediate oxygen tension (*e.g.*, a few millimeters from an air bubble). Metabolic studies reveal that each of these tactic responses can be correlated with a decrease in the rate of carbon assimilation (through photosynthesis, respiration, or both). Tactic responses do not occur under conditions, such as absence of substrate, which preclude anabolism. The saturating light intensity for phototaxis in the presence of propionate is about four times as high as that in the presence of other common substrates (acetate, pyruvate, succinate, etc.). The metabolism of propionate probably begins with a carboxylation to yield succinate; carbon dioxide is needed to initiate the respiratory dissimilation of propionate by *R. rubrum.* Accordingly a compound important in the mediation of tactic responses should be sought among the substances involved in the carboxylation of propionate. One possibility is that the labile disulfide bond of thioctic acid is involved in the mediation of phototaxis, in analogy with recent speculations concerning visual excitation.

Molecular weight of hemerythrin of Phascolosoma gouldi by x-ray diffraction. WARNER E. LOVE.

Hemerythrin contains iron, but not heme, binds oxygen and is found in nucleated erythrocyte-like cells in the coelom of the gephyrean worms. The pigment from *Phascolosoma gouldi* crystallizes on dialysis in the cold against 20% ethanol usually as long laths, rather thin for x-ray diffraction; however one batch crystallized in a more suitable tabular form.

Examination of the wet crystals by the oscillation and precession methods showed them to be monoclinic, space group $P2_1$, with $a = 56.9$ Å, $b = 134.6$ Å, $c = 74.2$ Å, and $\beta = 81°36'$. Therefore the unit cell volume for wet methemerythrin is 562,000 Å^3.

The volumes of single large perfect crystals were calculated from their linear dimensions, which were measured with an ocular micrometer. Each crystal was then dissolved quantitatively to a known small volume and the absorption at 278 mμ observed. Having previously determined the specific extinction coefficient (2.75 mg.$^{-1} \times$ cc. $\times$ cm.$^{-1}$), the weight of dry protein per unit volume of crystal (0.71 mg. $\times$ mm.$^{-3}$), and the weight of protein per unit cell (3.97×10^{-19} gm.) were calculated. The molecular weight of the asymmetric unit (the space group demands two per unit cell) is 119,000.

Iron and oxygen analyses showed oxyhemerythrin to be 0.88% Fe and gave a measured value of 1/2.42 for O_2/Fe. Thus there are 18.9 Fe atoms and 7.8 O_2 molecules per asymmetric unit, or, using nearest integers, 8 O_2 for 19 Fe. Although no protein crystal is yet known in which the asymmetric unit is two or more molecules, the possibility must be considered. If the molecular weight were half 119,000, O_2/Fe would be either 4/10 or 4/9. These ratios are considered a little too far from the measured value. In addition the sedimentation velocity of Resnik and Klotz fits somewhat better the value of 119,000.

Pteridine coenzymes in one carbon metabolism. BARBARA E. WRIGHT. No abstract submitted.

Secretion of inert gases and oxygen by the swimbladder of fishes. P. F. SCHOLANDER, L. VAN DAM AND THEODORE ENNS.

The swimbladders of living whitefish (*Coregonus*), caught in Lake Michigan, were found to contain some 99% unabsorbable gas, confirming earlier findings by Hüfner in 1892 and by Saunders in 1954. The gas content measured in the bloated fish would have given a neutral buoyancy at a depth of 100 meters. Therefore the gas was produced against a pressure gradient of 10 atmospheres. The identity of this gas has been determined in a series of fishes. It contained less than 0.1% combustible gases, and mass spectrometric analyses showed that the argon-to-nitrogen ratio was near to that in air. The high nitrogen pressure is therefore produced by some non-chemical means which also concentrates argon.

Codfish treated with yohimbine after complete deflation were kept in water containing O^{18}-labeled oxygen. In ten hours the swimbladder filled with oxygen, all of which was labeled. Hence the secreted oxygen is derived directly from the molecular oxygen dissolved in the sea water.

Gas secretion from the intact gland has been observed in laboratory experiments. When secreting oxygen the gas gland in barracuda was bright arterial in color, and cyanotic when not secreting. The A-V difference in the blood entering and leaving the gland was low (1–2 vol. % or less), and the oxygen-combining capacity of the leaving blood was not measurably lowered. Gas bubbles secreted under a Saran film by the intact gas gland of cod contained 5–16% CO_2 and 30–81% oxygen.

AUGUST 2, 1955

Motion pictures of cellular changes in tadpoles following x-ray irradiation.[1] CARL CASKEY SPEIDEL.

Tadpoles of *Bufo, Rana* and *Amblystoma* were subjected to whole-body x-irradiation with single doses ranging in 16 strengths from 200,000–250 roentgens. A dose of 100,000 r was sufficient to kill within 55 hours, 25,000 r within 11 days, and 10,000 r within 23 days. A dose of 1,000 r or less was ordinarily not lethal for the 58-day period of the observations. Tadpoles dying during the first two days were often markedly edematous; those dying after periods of 1–4 weeks following less severe doses were usually extremely emaciated. Respiration, circulation, excretion, and behavior in swimming and feeding were noticeably affected.

Histological changes in cells and tissues were observed *in vivo* and recorded by cine-photomicrography. The following scenes were obtained from irradiated tadpoles after periods varying from two hours to three weeks: early circulation reaction with marked lagging of circulatory leukocytes and increased rate of diapedesis; damage to some leukocytes, erythrocytes, and blood vessels; extravasation of blood; vacuolation of endothelium; occlusion of small vessels; leukopenia; excessive sticking of erythrocytes at vessel forks; injury to some nerve fibers with early appearance of fluid between myelin sheath and axis cylinder and later degeneration; lateral-line organ atrophy; mild injury to some muscle fibers with transformation of several sarcomeres into retraction clots at muscle-tendon zones; also emaciation, vacuolation, wrinkling, and complete degeneration of some fibers; injury to the epidermal epithelium with mal-alignment of cells; contrast in melanophore behavior in normal and irradiated animals; contrast in leukocyte reaction in normal and leukopenic irradiated animals; examples of tissue destruction in irradiated tadpoles by an invading ciliated protozoan, *Tetrahymena.*

Protoplasmic streaming in amoeba. ROBERT D. ALLEN.

As an amoeba advances, its tail shortens with respect to a point of reference on the side. This has been interpreted as a contraction of the ectoplasmic tube to force the passive endoplasm forward. The possibility should not be overlooked that the endoplasm may be self-propelled and may drag the tail. The following experiment emphasizes this possibility.

An amoeba was washed in Pyrex-distilled water and drawn intact into a quartz capillary (D. was 10–50 microns for *Amoeba proteus* and 40–100 microns for *Chaos chaos*), which was placed on a slide in a pool of immersion oil. These elongated amoebae advanced monopodally or exhibited "fountain streaming" without progression or tail shortening. The capillary was broken with a knife on both ends 10–30 microns peripheral to the cell membrane, which thereupon ruptured, permitting the free protoplasm to mix slowly with the water on both ends. Soon ecto- and endoplasm could not be distinguished and vigorous streaming took place in all of the protoplasm at a rate often exceeding that in the intact cell. "Streams" of protoplasm passed one another moving in opposite directions. Each "stream" behaved as a structural unit (judging from the behavior of its included granules) undergoing peristaltic and twitch-like contractions of an irregular nature. In the presence of traces of calcium, contraction of the "streams" squeezed out a fluid to form hyaline vacuoles. With the hyaline material unavailable for resorption, the streams contracted irreversibly. However, in the absence of added calcium, free amoeba protoplasm exhibited vigorous streaming for up to 70 minutes. During this period, streams initially 40–50 microns wide often were seen to tear like fabric along their long axes to form ever narrower streams, sometimes finally containing only a single row of granules. Similar results were obtained when endoplasm was sucked out of the front of an advancing amoeba into a capillary.

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[1] This investigation was supported by a research grant (PHS B-359C) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.

These results throw some doubt on the importance of the cell membrane, the shortening tail, and the ectoplasmic tube as ultimate factors in protoplasmic streaming in amoeba, although the entire cell is, obviously, required for normal amoeboid progression.

Enzymic synthesis of a new dinucleotide by a novel method of biosynthesis. S. G. A. ALIVISATOS.

Recently D. W. Woolley suggested that a new biosynthetic mechanism may exist. The energy for such synthetic processes is derived from the destruction of linkages in which an "onium" configuration participates. The present work was undertaken jointly with Dr. Woolley.

Coenzyme I is such an "onium" salt and it was used in an attempt to synthesize enzymatically new dinucleotides. The enzyme was beef spleen DPNase solubilized by the combined action of desoxyribonucleic acid and iso-amylalcohol. The enzyme was further purified by ammonium sulfate fractionations, alumina C_γ and calcium phosphate gel adsorptions and elutions with phosphate buffers.

The purified enzyme was found to catalyze the following reaction:

DPN + 4-amino-5-imidazole-carboxamide →
(4-amino-5-imidazole-carboxamide)-ribose-phosphate-phosphate-ribose-adenine + nicotinamide + H^+

The reaction is irreversible and about 8200 calories of free energy of hydrolysis of the nicotinamide-ribose bond in the molecule of DPN are utilized during this synthesis.

The new dinucleotide was isolated in relatively pure form and its chemical structure was established. The U.V. spectra and the spectra for the azo-derivative with N-(1-naphthyl)-ethylene diamine have also been obtained. Upon action of organic pyrophosphatase the new dinucleotide yields AMP and 4-amino-5-imidazole-carboxamide ribotide. The latter is identical with the mononucleotide isolated by Greenberg and by Buchanan. The new dinucleotide may be a substrate for further incorporation of a 1-carbon unit and closure of the purine ring. The product of such a reaction would be a pyrophosphate of inosinic and adenylic acids.

This alkylation of the imidazole is different from base exchanges of DPN with isoniazide and other pyridine bases. These base exchanges involve no loss of the quarternary character of the linkage of the base with ribose in the molecule of the dinucleotide, they are not accompanied by H^+ production and they presumably involve little energy change.

Structure and function of the ligament of Pelecypoda. PAUL S. GALTSOFF.

In Pelecypoda the ligament is a band of elastic protein which joins the valves at the dorsal edge of the hinge. Its function is purely mechanical. As the adductor muscle relaxes, the compressed material of the ligament expands and pushes the valves apart. The ligament itself is not contractible. It usually consists of one central portion acting as a spring and two lateral ones which in closed condition are under tensile strain.

The ligament is a highly complex structure. It consists of concentric rows of arches with the bands of fibrillar material between them. These two structural elements repeat themselves with regularity varying only in the width of their respective layers. The arches are made of rows of tightly packed oval-shaped globules embedded in a homogenous substance. Their arrangement coincides with the lines of elastic stresses and suggests that the arches act as springs. The fibrillae are oriented at about a 45° angle to the arches. They may be compared to coils which exert additional elastic force when the ligament is compressed.

Examination with the electron microscope shows that the fibrillae are from 450–500 Å. in diameter. On a longitudinal section the ligament appears as a membrane honeycombed by holes from 500 to 600 Å. in diameter. Tentative conclusion is that the fibrillae are tubular. The ligament is a nonliving structure secreted by the tall and slender epithelium cells of the subligamental ridge. Chemically it is considered to be identical with conchiolin—a scleroprotein of the organic matrix of the shell. The complexity of its structure reflects the differential rate of secretion of the material deposited in accordance with the mechanical demands of the organism.

August 9, 1955

Effects of ultraviolet on regenerative activity in urodeles. E. G. Butler and H. F. Blum. No abstract submitted.

Study of intracellular potentials in pacemaker and integrative neurons of the lobster cardiac ganglion.[1] Susumu Hagiwara and Theodore H. Bullock.

Selected neurons of the nine in the virtually isolated ganglion of *Panulirus interruptus* have been penetrated by a microelectrode. Resting potentials are about 60 mv. and last for hours. Patterned bursts of activity synchronized with the other cells (recorded by external electrodes) occur without deterioration for long periods. Each large cell burst begins with a sudden 20 mv. depolarization on whose slow falling phase a series of small, slow deflections are superimposed. From the summits of some of these deflections, spikes of 5–15 mv. appear. Because each small, slow deflection is preceded by a small spike in the external recording leads, probably from one of the small cells which act as pacemakers, we regard them as synaptic potentials. Spikes originate from them but only at some distance down the axon, hence are seen in the cell as a small electrotonic fraction. Antidromic spikes similarly do not invade the cell but nevertheless reach and reset the pacemaker in spontaneous neurons. A single synaptic potential elicited by one presynaptic impulse can have up to five spikes on its 40 msec. declining phase. This is explained by the asymmetrical relation between soma and axon: the slow fall of soma potential permits its electrotonic effect to be felt strongly at some distance in the axon whereas the rapid course of the spike prevents its effect from being felt as far, protecting the synaptic potential from cancellation by the first spike. Spatial summation is absent in large follower cells. Repetitive presynaptic stimulation results in declining amplitude of synaptic potentials, proportional to frequency; this is important in burst patterning. Pacemaker cells rhythmically gradually depolarize and then fire. Additional depolarization, presynaptic or artificial, results in postexcitatory depression, hence a pause. Bursts can thus be patterned even if some followers have independent automaticities.

Further study of the giant synapse in the stellate ganglion of squid.[1] Theodore H. Bullock and Susumu Hagiwara.

Microelectrodes have been placed inside the large (100 micra or more) presynaptic and postsynaptic fibers within two millimeters, sometimes within one millimeter, of the synapse. Spikes are like those in ordinary axon. Synaptic potentials were obtained by repetitive stimulation of the prefiber at a critical frequency which maintains a level of fatigue just below spike threshold. At this distance from the synapse in the postfiber, spike threshold is between one and ten mv. The synaptic potential rises in about two msec. and falls in three at 15° C. The fall is not exponential. There is an undershoot. Fatigue not only reduces rate of rise but shortens the duration of rise and slows the fall. A true delay remains which cannot be accounted for by conduction in the last millimeter. This is confirmed by extracellular steel microelectrodes in the ganglion. Values of one to two msec. near 15° C. are insensitive to the fatigue imposed. As long as it was possible to believe there is no real delay, it could not be expected that the prespike would be distinguishable in a postfiber electrode. But now that such a delay is verified, the prespike must be separately discernible and the question becomes how large is it? Our postfiber recordings at high gain show a deflection attributable to the prespike which is less than one mv. (after correcting for a reasonable spatial decrement) across the synaptic membrane. This is far too small to stimulate a postjunctional response, by comparison with direct electrical stimulation through the internal electrode. It is concluded that current cannot be the synaptic transmitter.

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[1] Aided by a grant from the National Institutes of Health.

The synaptic potential does not add to or alter the form of the antidromic spike arriving at the same time or just before. The first effect is an addition of a small deflection (internal positivity) to the undershoot. The synaptic potential is not enhanced in proportion to hyperpolarization. These properties are in contrast to other junctions.

The electrocardiogram of the alligator. CHARLES G. WILBER.

Cardiological studies in the intact *Alligator mississippiensis* have not been made to any extent. The present work describes the normal electrocardiogram of the alligator and the changes effected by elevated body temperature. Preliminary results indicate that the wave of excitation passes over the heart in the following order: sinus venosus, right atrium, left atrium, base of ventricles, apex of ventricles. The pacemaker is located in the wall of the sinus venosus near the sinu-atrial junction. Values for the normal ECG taken at 22° C. are as follows: 40 beats/minute; P–R, 0.40 second; QRS, 0.04 second; QR, 0.12 mv; T, 0.24 second and 0.05 mv; Q–T, 0.06 second. If the whole animal is exposed to elevated temperature, the colonic temperature rises and the heart rate increases. The latter increase is not properly described by a single straight line; at about 34° C. the rate increases more slowly than between 22° and 34° C. Above 40° C. the heart becomes disorganized and irreversibly damaged. T wave duration decreases from 22° to 33° C. above which it increases rapidly. This indicates that with increase in temperature there is first an increase in oxidative metabolism in the heart, but that above 33° C. a rapid inhibition of oxidative metabolism obtains. The Q_{10} for the heart rate between 25–35° C. is 1.5. Since most of the data in the literature are based on isolated hearts, with Q_{10}'s of 2, the differences suggest that the activity of tissues in the nonmutilated animal may not be the same as in tissues removed from their proper physiological environment.

AUGUST 16, 1955

Differences in response of x-rayed eggs and spermatozoa of Habrobracon to anoxia. ANNA R. WHITING AND WILLIAM R. MURPHY. No abstract submitted.

High frequency of transduction of genes by bacteriophage in Salmonella. H. H. PLOUGH AND MARGARET ROBERTS.

One of the strains of *Salmonella typhimurium* (549) which is being used in our laboratory for genetic studies is lysogenic, that is, it carries a temperate bacteriophage in the prophage stage. Occasional cells are lysed releasing free phage particles which can be assayed on an indicator strain of Salmonella (533) which then becomes lysogenic also. When the bacteriophage designated P 549 is transferred to a radiated substrain of 533 requiring histidine and leucine, colonies can be isolated which have reverted from H^- to H^+ or from L^- to L^+. These are typical cases of transduction as described by Lederberg, Zinder and others. They occur one at a time, and never occur spontaneously, nor do they occur if a strain of 549 which is itself H^- or L^- is used as the phage donor. The distinctive character of these cases is the high frequency of transduction per phage particle, approximately one H^+ transduction for every thousand free phage particles. This is an efficiency nearly a thousand times greater than has been previously reported. The frequency per cell is about the same as reported; namely, one in about 10 million cells is transduced. Electron microscope photographs of the phage adsorbed on the Salmonella cells were shown. They are similar in appearance to the larger particles of the T-even series in *E. coli.*

Paralysis in double mutants of Chlamydomonas. RALPH A. LEWIN.

Chlamydomonas moewusii is a biflagellate unicell, haploid, heterothallic, and morphologically isogamous. The mating-types are arbitrarily designated as *plus* and *minus*. Pairs *in copulo* swim by the activity of the *plus* partner, the flagella of the *minus* cell being inactive after mating. Following U–V irradiation, many mutants have been obtained which, though flagellated, are paralyzed. Twelve of these have been studied genetically, and no case of allelism has been found, indicating that at least 12 distinct loci are involved in normal flagellar activity. Some of these mutants are capable of recovery when paralyzed *plus* cells are paired with wild-type *minus* gametes. Motility can also be restored when such "recoverable" mutants are paired with paralyzed cells bearing a different gene for paralysis. These effects can be attributed to the transfer of an essential motility factor through a bridge which joins the copulant protoplasts (Lewin, R. A., J. Gen. Microbiol., *11:* 358–363, 1954).

By genetic recombination, clones have been obtained bearing two dissimilar genes of the recoverable paralysis type within the same haploid genome. Such paralyzed double mutants recover motility when paired with wild-type or with a third mutant, but not when paired with either parental mutant type.

Two basic hypotheses could account for these facts.

(1) A number of independent processes lead to the synthesis of motility factors: the absence of any of these results in paralysis.
(2) A number of consecutive steps lead to the synthesis of a single motility factor: a genetic block at any step results in paralysis.

Obviously, both kinds of mechanism could be involved simultaneously in a smaller number of stepwise syntheses. Means are being sought by which these hypotheses may be tested.

Physiological races of the diatom, Navicula pelliculosa. JOYCE C. LEWIN.

The classification of diatoms is based on the size and shape of the silica wall, of which the detailed patterns provide highly characteristic specific differences. Ten separate isolates of *Navicula pelliculosa* have been obtained from various localities in Connecticut, Bermuda, Alaska, Ontario, and Northwest Territory. Although the frustle patterns of these isolates appear identical in electron micrographs, the strains in culture show differences in colony form, color, osmotic tolerance, and growth at different temperatures. The existence of physiological races should be recognized in ecological studies of diatoms.

AUGUST 23, 1955

Some observations on the incorporation of glycine-2-C^{14} into amphibian embryos. PHILIP GRANT.

Recent demonstrations of a "cytoplasmic DNA reserve" in *Rana pipiens* eggs have suggested a hypothesis for the existence of two mechanisms of DNA synthesis during development. Cleavage up to late blastula, when little or no new increase in DNA content per embyro occurs, could be characterized by the *absence* of a de novo synthesis of DNA from low molecular weight precursors (glycine, CO_2, etc.), and a preferential utilization of "cytoplasmic DNA" for the formation of new nuclei. After gastrulation, it is suggested that the de novo synthetic mechanism is established and proceeds as a major pathway during later development.

This hypothesis was tested by comparing the per cent incorporation of injected glycine-2-C^{14} in fertilized eggs (allowed to develop for 95 hours, samples of different stages being taken for analysis) with incorporation into gastrulae, similarly treated. Short term rate experiments for five different stages were also performed. A modified Schmidt-Thannhauser technique was used to obtain the DNA as well as the following additional fractions: acid-soluble, lipid, protein, RNA and CO_2. This permitted a comparison of the "balance sheet" of glycine metabolism for different developmental stages.

The results obtained permit the following minimal conclusions: 1—the pattern of glycine incorporation into all fractions is markedly more active in stages after gastrulation than before; 2—a de novo synthesis of DNA from glycine *does* occur prior to gastrulation; however, it represents approximately 1/10 to 1/20 of the DNA required for new nuclei. Presumably, the remainder is provided by the cytoplasmic reserve; 3—after gastrulation, glycine incorporation into DNA is calculated to provide approximately 25% of the new DNA, a several-fold increase, which is taken to suggest a mobilization of a system when developmental requirements demand it.

Fucose metabolism in Escherichia coli. MAURICE GREEN. No abstract submitted.

The potentiation by narcosis of the beneficial effects of hypothermia in asphyxia of the neonatal guinea pig. JAMES A. MILLER, JR. No abstract submitted.

Action of a thrombocyte protein on capillary permeability. W. WILBRANDT AND P. LUESCHER. No abstract submitted.

GENERAL SCIENTIFIC MEETINGS

AUGUST 29–SEPTEMBER 1, 1955

Abstracts in this section (including those of Lalor Fellowship Reports) are *arranged alphabetically by authors* under the headings "Papers Read," "Papers Read By Title" and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Coordinated ciliary movement on the gill of the ribbed mussel, Modiolus demissus plicatula. EDWARD L. AIELLO.

Cilia on the lateral epithelium of the gill filament beat in such a way that successive waves of activity, when viewed from the frontal surface of the filament, pass dorsally on the left side, ventrally on the right. The cilia are arranged in rows running obliquely across the epithelium and beat in line with these rows so that the effective beat is obliquely downward between the filaments, whereas the wave front is directly across the epithelium. This arrangement obtains on all eight lamellae. The rows of cilia are about 0.5 micron apart; no inter-connections have been observed. Cinematography shows that velocity and wave-length vary slightly even during one second for a given area and differ greatly from one area to the next on the same filament at the same instant. Frequency and velocity increase with temperature from 7 to 35° C., the experimental limits, and equilibrate to sudden changes at comparable rates. After naturally occuring local inhibition, beating resumes in phase with non-inhibited areas, indicating the retention of coordinating activity during beat inhibition. The same is true during slow stoppage. Long waves often separate into two, suggesting that coordination is not due merely to a series of impulses. Synchronous beating was not observed; asynchronous beating was observed rarely and usually either stopped or became metachronal within a few seconds. Although these and other observations suggest a coordinating activity distinct from the motile activity, none of them directly rules out the possibility of autonomous beating brought into a definite phase relationship by mechanical and chemical factors resulting from the beating itself, and then, once set this way, retaining this relationship through short periods of inhibition and when stopping.

Pigment migration in partially fertilized Arbacia eggs. ROBERT D. ALLEN AND EDWARD C. ROWE.

In eggs of the sea urchin, *Arbacia punctulata*, one of the first evidences of activation after the cortical reaction is the migration of red echinochrome-containing granules from the cell interior to the periphery to form a new sub-surface granular layer.

In the eggs of *Arbacia* and of several other sea urchins, it has previously been found that the cortical reaction could be interrupted by stretching the egg surface in a glass capillary, warming the egg mildly or pretreatment with nicotine. Any of these treatments produce partially fertilized eggs in which activated and unactivated cortex coexist in the same egg, and the activated cortex causes only its underlying cytoplasm to commence development. In partially fertilized *Arbacia* eggs, pigment granules migrate only to the surface at which the cortical granules (Harvey, Moser) have broken down. The concentration of pigment granules at the fertilized surface of partially fertilized eggs is greater than that at the surface of entirely fertilized eggs, suggesting that the fertilized surface recruits pigment granules from the surrounding cytoplasm. This was especially clear in the case of partially fertilized eggs obtained by stretching the surface in a quartz capillary and warming the egg slightly during the cortical reaction by removing the heat filter of the microscope lamp (about 26° C.). Pigment migrated normally to the fertilized, but not to the unfertilized, surface. After half an hour, there was a dense accumulation of pigment at the border between fertilized and unfertilized cortex. Beneath the adjacent unfertilized cortex was a 25-micron wide unpigmented zone from which the pigment granules had been recruited. Pigment migration in the *Arbacia* egg must then be a specific interaction between fertilized cortex and the pigment granules, whether they be in fertilized or unfertilized cytoplasm. The maximum attraction distance is about 25 microns in partially fertilized eggs.

Effects of the level of nutrition on the growth pattern in Campanularia colonies.[1] SEARS CROWELL, CHARLES WYTTENBACH AND FRED WILT.

The manner of growth of colonies of the hydroid *Campanularia flexuosa* is such that there are localized zones of proliferation and of prospective proliferation. By varying the quantity of food (*Artemia*) given to young growing colonies their pattern of growth has been modified. This modification results from the fact that with partial starvation certain zones are practically unaffected, others much delayed in the time at which active proliferation begins.

The total mass of colonies is closely proportional to the quantity of food received. As compared with well fed colonies, partially starved ones show these differences: the tip of the main stolon and the origin of new stems near this tip are only slightly retarded. The opposite end of the main stolon fails to proliferate or does so more slowly. Lateral stolons are fewer. Stems have fewer hydranths and are shorter. The older the stem the more it shows this effect. The rate at which pedicels develop into hydranths is unaffected. However, the zone of prospective proliferation which can produce a new pedicel and hydranth is much later in becoming active or, in extreme cases, fails completely. Branches of the stems and gonangia are lacking. Hydranths undergo regression earlier.

We conclude that active growing zones have priority for the reduced nutritive pool, and that, if they grow at all, they do so at or close to the maximum rate. Among the zones of prospective growth there is an order of priority approximately as follows: tip of the main stolon; zone behind the tip of the stolon which produces a new stem; zones on stems which will produce the next hydranths, and these in order of the age of the stem; opposite tip of the main stolon; lateral stolons; gonangia and branches on the stems.

A study of the distribution of homarine (N-methyl picolinic acid).[2] E. L. GASTEIGER, JOHN GERGEN AND PAUL HAAKE.

The quaternary ammonium compound, homarine, has been found in large concentrations in invertebrate tissues, but its function is yet unknown. In the hope that clues to its function could be uncovered, a study of its distribution with respect to tissue and phyla was undertaken. The homarine content of crude tissue extracts was quantitated by means of their U.V. absorption spectra. The accuracy of this simple method was checked by fractionating numerous extracts on a resin column of Dowex 50 acid resin, × 8. Extracted homarine was essentially identical with synthetic homarine on the basis of its absorption curves and its behavior on the column. Homarine was found in the following phyla at the indicated maximum concentrations (mg./gm. wet weight): Porifera, less than 0.2 (not conclusive); Coelenterata, 0.6; Ctenophora, 0.2; Annelida, 1.1; Mollusca, 7.7; Echinodermata, 0.3; and Arthropoda, 8.7. There was no evidence by our methods for its presence in Vertebrata or in fresh water species of Mollusca and Arthropoda. Within the phylum Mollusca, Venus and Pecten tissue content was considerably below (less than 1.5 mg./gm.) that of Loligo, Busycon and Nassa. Careful examination of Homarus and Loligo revealed a wide distribution throughout the tissues with higher concentrations in ganglia and muscle of lobster and in ganglia and liver of squid. The sharp contrast between fresh water and salt water species suggests an osmoregulatory function for homarine.

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[1] This investigation was supported by a research grant (H-1948) from the National Heart Institute, of the National Institutes of Health, Public Health Service and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[2] Research supported by the Atomic Energy Commission.

Existence of a "resting potential" in the egg of the starfish Asterias forbesii.

H. GRUNDFEST,[1] C. Y. KAO, A. MONROY[2] AND A. TYLER.[3]

Several investigators have attempted to determine whether or not a potential difference exists between the inside and outside of the eggs of various echinoderms and fish. All have consistently reported its absence. A recent careful determination (Rothschild and Barnes, 1954) of Na, K and Cl contents of the egg of a sea urchin shows the high K and low Na and Cl (with respect to the medium) typical of excitable tissue. This and the fact that it is difficult to be certain that the micro-electrodes actually penetrate the egg (see Tyler and Monroy, 1955) warranted re-examination of the problem.

It proved possible to penetrate eggs of Asterias with one or even two micro-electrodes by the technique described by Tyler and Monroy (1955). When the electrode ($< 0.5\ \mu$ tips and containing 3 *M* KCl or 0.3 *M* KCl) is jarred through the plasma membrane the potential change is immediately observed on the oscillographic trace. This has given transient values of 60 millivolts (inside negative) and stable values at 30 to 40 millivolts. It can be assumed that the higher figure is nearer the true value because of the possibility of leakage (short-circuiting) around the electrode. As the K content of the surrounding fluid is increased the potential drops towards zero, and returns to its original value when the egg is again surrounded by normal sea water. When the electrode is removed the potential drops again to zero. The eggs are fertilizable while the electrode is inside or after its removal.

Determination of the K concentration in the water of Asterias eggs shows it to be 17 to 19 times that of sea water and Na to be very low within the egg.

By placing a second electrode within the egg and passing a current through, it has been possible to measure at the same time membrane resistance and capacitance, which are 2000 to 3000 ohms-cm^2. and ca. 0.5 to 1.0 microfarads per cm^2., respectively.

Relationship of mechanical to resting potential changes in contracting muscle.

RITA GUTTMAN AND JOHN A. DOWLING.

It has already been established that if *Mytilus* smooth muscle is rapidly cooled after treatment with sea water containing from three to seven times as much KCl as is ordinarily found in sea water, there occurs a large change in resting potential in the positive direction, and the muscle contracts. Potassium and rapid cooling were used merely as devices to investigate the relationship between potential changes and the mechanical aspects of contraction.

Apparently contraction occurs only when a critical P. D. change is brought about (7.0 ± 2.5 mV or about 38% of the original value). Rapid cooling alone (by as much as 23° C.) without previous treatment with extra potassium results in a P. D. change less than this critical amount and so does not result in contraction.

Low sodium, high sodium, calcium and magnesium may not be substituted for potassium in this phenomenon. However, rubidium, in concentrations even lower than the potassium used, is effective. Thus the potassium effect is not a completely specific one.

[1] Supported by a grant from the Marine Biological Laboratory under its ONR Research Contract (Nonr-09703).

[2] Fulbright Fellow.

[3] Supported by a grant (C–2302) from the National Cancer Institute, Public Health Service.

It is doubtful whether rapid cooling exerts its effect by influencing metabolism. The phenomenon described still persists when the muscle is treated with nitrogen, or with sodium iodoacetate or sodium cyanide in fairly high concentrations.

The effect of Versene on the structure of plant chromosomes. BEAL B. HYDE.

Recently Mazia has shown that chromatin can be dispersed in water after mild treatment with Versene (disodium salt of ethylene diamino tetracetic acid). Steffensen has shown an increase in spontaneous chromosome breakage in calcium-deficient plants and Levine has observed an increase in crossing over in Versene-fed Drosophila. As a result it has been suggested that calcium or possibly some other divalent cation forms essential links between units of chromatin or molecules of DNA. To determine the immediate effect of a cation deficiency on chromosome structure non-excised root tips of onion and *Vicia faba* were treated with .01 M solutions of Versene. The tips were fixed in a 3 : 1 alcohol-propionic acid mixture. Squash preparations were stained with a saturated solution of Sudan Black B in 45% propionic acid. Preliminary tests have shown that this stain is not effective for chromosomes after such treatments as Feulgen hydrolysis or lengthy alcoholic fixation.

The first noticeable effect (within three hours) of the treatment is suppression of anaphase movement of chromosomes. Accompanying this change is a marked swelling of metaphase and late prophase chromosomes. The swelling is not uncoiling. The chromosomes take on an even reticular appearance. The fact that swelling is not accompanied by any marked loss of stainability accords with previous findings that Versene treatment breaks chromatin into smaller units—not into its components such as DNA, histone, etc. The swelling process will continue until discrete metaphase chromosomes can no longer be distinguished. Resting nuclei lose all structure to become uniformly fine granular.

To determine whether calcium is the critical ion chelated by Versene in these treatments, root tips were treated with a 1 : 1 molar ratio of $CaCl_2$ and Versene. These continue to divide normally. A similar solution of $MgCl_2$ and Versene produced typical swelling and subsequent dispersion.

Seasonal resorption and restoration of the copulatory organ in the mud snail, Nassa obsoleta.[1] CHARLES E. JENNER AND NORMAN A. CHAMBERLAIN.

A collection of large *Nassa obsoleta* from the shallow water near the mouth of Great Pond, Falmouth, Massachusetts, on June 14, 1955, consisted of 45 males and 52 females. Twenty-eight of the males had normal sized copulatory organs; in the remaining 17 this structure was reduced to a small papilla (62% of males). By dissection it was found that 13 of the latter group were parasitized by trematodes (about 76%) whereas in a sample of 20 males having normal copulatory organs, none was found to be parasitized. Thus it is clear that in this case, penis size reduction is at least partly related to parasitism, suggesting to a degree a causal relationship.

The per cent of males with fully developed copulatory organs occurred in collection on June 25, July 2, July 7 and July 14 as follows: 45%, 24%, 5% and 4%, respectively. This progressive reduction in the per cent of males with full sized copulatory organs was closely correlated with the cessation of seasonal reproduction as shown by the inability to find egg cases in this area after this period, whereas they were common before, and the reduction to near zero of the number of snails with gametes in the genital tracts. By following the change in the per cent of males having resorbed copulatory organs it is possible to describe the cessation of seasonal reproduction in this species in a very precise, quantitative manner. This provides a useful method of comparing populations from different areas in regard to termination of the reproductive period.

Available information indicates that *Nassa obsoleta* does not start its seasonal reproduction until sometime after March. It was, therefore, surprising to find that by August 10, 1955, 93% of the males from a collection of 60 from Great Pond possessed fully developed copulatory organs.

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[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C3).

Graded response in squid giant axon. C. Y. KAO AND H. GRUNDFEST.[1]

Graded response in nerve is normally superseded and masked by the explosive all-or-none spike. In fact, the all-or-none response in nerves has long served as a model of response in all excitable tissues, in spite of evidence to the contrary in motor end-plates, electroplaques, synapses and dendrites, and invertebrate muscle fiber.

In squid giant axons, graded responses have been elicited by exciting during refractoriness or after microinjection of certain drugs (eserine, prostigmine, d-tubocurarine, acetylcholine, butyrylcholine, and carbaminycholine). Action potentials produced unidirectionally by stimulating successively at one end, or collisionally at both ends, have been simultaneously recorded at two or three intracellular sites. Immediately at the cathode, refractory responses may reach a level with overshoot, or approaching that of the conditioning spike without producing propagated action potentials. At a fixed stimulus interval, such responses undergo a wide range of amplitudes, depending on stimulus strength, and spread for short distances decrementally. As recovery from refractoriness proceeds, regions distal to the stimulating cathode regain the all-or-none mechanism abruptly with rapid increases in response amplitude, while the cathodal region may not undergo any or only small increments. Propagated responses may also result from activity in more excitable intermediary regions caused by a decremented potential which has spread electrotonically from the cathodal region.

Graded response mechanism is also unveiled by microinjecting drugs in localized regions. By varying the density of the local circuit current during collisional responses, the affected region can produce smoothly graded increases in potentials of 20–30 mv. above the unidirectional response. Graded action potentials are accompanied by graded impedance changes. Although usually masked by the all-or-none spike, the graded response appears as the alternate under circumstances in which the former is inoperative, probably via a response pathway which is not completely identical to that underlying the all-or-none response.

Solubilities of denatured bovine plasma albumin. MILTON LEVY AND ROBERT C. WARNER.

The chemically ill-defined processes of denaturation lead to decreased solubility of proteins during heating. Using pH and salt concentration as variables many variations of the solvent leave the native protein in solution. Choice of one of these to test the solubility after heating is arbitrary. We have systematically explored the solubilities of native and heated (pH 7.0, 65.7°, ionic strength 0.2) bovine plasma albumin in ammonium sulfate and sodium trichloroacetate solutions, buffered with acetic acid, sodium acetate mixtures, by the variable solvent method (salt concentration varied). The solubility of the native protein is a monophasic function of salt concentration at constant pH. The solubility of heated protein is a biphasic function. As time of heating increases a fraction (P_3) precipitates at lower and lower salt concentrations increasing in amount, rapidly at first and then more and more slowly. Another fraction (P_2) behaves to the solubility test like native protein. It decreases in amount at a very rapid rate initially but very slowly later on. These changes are demonstrable with ammonium sulfate at pH 4.9 or 5.4 and with trichloroacetate at pH 4.15. The fractions P_2 and P_3 were isolated by salting out and retained their properties. P_2 is much more slowly converted to insoluble material on heating than is native protein. P_3 on heating becomes even less soluble than before and forms no detectable amount of P_2. Fractionation of native protein with ammonium sulfate indicates that P_2 is not a heterogeneity in the native material. It appears that an initial rapid reaction in heated bovine plasma albumin solution yields at least two products, one of which polymerizes rapidly and irreversibly whereas the other polymerizes only slowly.

Induced swelling of nuclei of Asterias eggs in the germinal vesicle stage. ALFRED MARSHAK.

From experiments with isolated nuclei, other investigators have reached the conclusion that the nucleus has no semipermeable membrane. In the present experiments it was found that nuclei retained within the cell could be induced to swell but under the same conditions, nuclei removed from the cells would not swell. Nuclear swelling occurred in the absence of the cell membrane, but a necessary condition for swelling was the attachment to the nucleus of at least a portion of the cell cytoplasm. It is concluded that nuclear swelling requires the functioning of structures physically connecting the interior of the nucleus with the cytoplasm and that these become altered or destroyed on removal of the nucleus from the cell. This conclusion is supported by observations of the nucleus made with the electron microscope.

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[1] Supported by a grant from the Marine Biological Laboratory under its ONR Research Contract (Nonr-09703).

No nuclear swelling resulted from treatment with aqueous solutions of 1,2 ethane diol and 1,2 propane diol, but there was pronounced swelling in solutions of 1,3 propane diol, 1,2,3 propane triol, and 1,2,3,4 butane tetrol, the most effective being the tetrol and the least effective the diol. Thus both the length of the carbon chain and the spacing of the hydroxyls determined the effectiveness of the compound in inducing swelling. Nuclear swelling induced by sucrose differed in that it took several hours to develop and was much smaller in extent.

Addition of ATP to solutions of glycerol prevented swelling. Dispersed cellular debris in the glycerol solutions was globular. When ATP (0.05%) was added to these suspensions, fibers appeared in abundance. ATP (0.05%) added to sea water produced a marked wrinkling and indentation of the cell surface but no visible alterations of the cytoplasm or nucleus. The observations suggest the presence of a contractile substance in these eggs with properties resembling those of myosin.

Formation of acrosome filaments in response to treatment of sperm with fertilizin in Asterias and Nereis.[1] CHARLES B. METZ AND JOHN B. MORRILL, JR.

Fertilizin treatment of sperm results in the appearance of a filament from the sperm acrosome. This effect was most strikingly demonstrated by Dan (1954) in a study on starfish. To confirm and extend Dan's observations the sperm of *Asterias forbesii* and *Nereis limbata* were fixed in 5% formalin and examined for acrosome filaments with the electron microscope. Acrosome filaments were observed in both Asterias and Nereis sperm following fertilizin treatment. Filaments were observed infrequently except in sperm from agglutinated suspensions.

A chelating agent is ordinarily required for fertilizin agglutination of starfish sperm. Accordingly, sperm in Versene were treated with fertilizin. In one experiment 33% of 30 such sperm possessed filaments. Sperm treated with Versene alone (12), fertilizin alone (75) or sea water alone (44) failed to show filaments. In three other experiments less than 3% of sperm treated with Versene alone revealed filaments. In one experiment 60% of fertilizin-treated sperm possessed filaments even in the absence of Versene. However, this fertilizin agglutinated sperm in the absence of Versene. The filaments observed were similar to those illustrated by Dan.

The normal Nereis acrosome is a conical structure of approximately the length of the sperm nucleus, the nucleus is slightly elongated and the tail extends freely. In one experiment both sea water- and fertilizin-treated sperm were of this type. In a second experiment in which the fertilizin-treated sperm showed strong macroscopic agglutination 85% of 48 fertilizin-treated and 30% of 90 control sperms showed a striking effect. The tails of these were coiled, the nuclei were round and shortened and the acrosomes consisted of fine filaments of uniform diameter. The over-all length of the nucleus plus filament did not exceed the normal. The figures suggest a retraction of nuclear and acrosomal material with the exposure of a central filament. They resemble very closely Lillie's (1911) drawings of Nereis sperm attached to the egg surface. Apparently the fertilizen-induced and spontaneous acrosome reactions of Nereis take a new and unusual form.

Further studies on the hatching of Fundulus heteroclitus. ROGER MILKMAN.

As previously reported, reversible inhibition of hatching in *Fundulus heteroclitus* may be accomplished by placing recently fertilized eggs in a sealed flask containing sea water and pure oxygen. Eggs so treated develop normally until hatching time; thereafter they remain without hatching and with little further growth for as long as a month. Washing with untreated sea

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[1] Aided by a grant from the National Science Foundation.

water for a few minutes is followed by initiation of the hatching process, which takes about 8 minutes at 24° C.

Oxygen's action is to promote the production of an inhibitory substance, directly or indirectly. Because only 20 mg. of eggs render 100 cc. of sea water inhibitory, excretory products were investigated first. Neither any of these nor other substances tried show the inhibition described. If the inhibitor is not an excretory product, its effective concentration must be small indeed, perhaps 10^{-7} or 10^{-8} molar.

The enzyme liberated during the hatching process which digests away the tough inner layer of the chorion is proteolytic, has an alkaline pH optimum, and does not clot milk. The inner layer is not digested by trypsin, however. Pepsin and hyaluronidase also have no effect.

Removal of the egg from the chorion is facilitated by the hatching enzyme. The indigestible outer layer is easily abraded away, and wherever this is done the underlying inner layer is digested in a few hours by water in which eggs have hatched.

The relationship of regenerative ability to the regression of hydranths of Campanularia. DONALD L. NATHANSON.

Individual hydranths in a colony of the hydroid Campanularia exhibit regression which is orderly and is dependent on the age of the hydranth. Crowell, who demonstrated this in 1953, suggested that the resorbed tissue is used by the colony. A clear proof for this could be obtained if it were demonstrated that a regressing hydranth, when isolated, could produce a new hydranth.

Hydranths of varied ages and stages of development were cut off at the base of the hydrotheca and kept in filtered sea water at 18° C. No food was given. In the best cases a new stolon develops at the cut surface, grows out, and gives rise to a new hydranth. Of eight hydranths cut off during regression, two formed a stolon and a hydranth; six formed only stolon. Of twenty normal hydranths carried through for several days, five produced stolons and hydranths, others only stolons. The originally normal hydranths did not, however, develop stolons until regression had occurred. Immature hydranths, cut off before emergence from the hydrotheca, may fail to differentiate further, or may develop into normal hydranths. In the former case they became altered to form a bulb-shaped sac whose cavity contains much resorbed tissue. A similar stage is present in normal hydranth regression. Six such bulbs produced stolons within three days. Seven immature hydranths grew to maturity three to five days after removal and three days later regressed. As the regression takes place, stolons and hydranths develop as described.

These observations show not only that regression of hydranths supplies a colony with material which can be used for further growth, but that if food is not available to the hydranth, regression is the only means by which material for further growth can be obtained.

A theory for the lifting of the fertilization membrane of the egg of Arbacia punctulata. A. K. PARPART AND P. C. LARIS.

Normal fertilization or artificial parthenogenesis leads to an explosive distintegration of many of the cortical granules of the egg of *Arbacia punctulata.* Cortical granules lie between the plasma membrane and vitelline membrane. Lifting of the vitelline membrane, the fertilization membrane, is due to the passage of water, accompanied by salts, from the surrounding medium into this perivitelline space. This uptake of water is due to liberation from the exploded cortical granules of colloidal material (presumably protein) which is for about half a minute after fertilization highly active osmotically. Two minutes after fertilization the particles of sub-microscopic size, which have osmotically lifted the vitelline membrane, begin to polymerize (coagulate) and in part add to the thickening of the original vitelline membrane and primarily to the formation of the hyaline layer.

The major items in the experimental justification of the foregoing theoretical statement of the cortical events following fertilization are as follows: (1) sea water solutions of 5% crystalline egg albumin or of 10% serum globulin do not quite prevent a little lifting; (2) one minute after fertilization, these same solutions in 0.5 and 1% concentrations cause a slight decrease in the amount of water in the perivitelline space, whereas (3) at 15 minutes a large decrease is induced; (4) the volume of the egg is the same at the time of complete formation of the hyaline layer as before fertilization; (5) eggs placed within one to two minutes after fertilization in 0.5 M NaCl do not form a hyaline layer; (6) the hyaline layer will solubilize in 0.5 M NaCl and will repolymerize if 0.01 Ca or Mg ions are added; (7) the submicroscopic particles of the hyaline layer will not pass through the fertilization membrane, though albumin molecules do. These observations were made on the television microscope.

Measurement of the growth of single cells with the interference microscope. L. M. Passano, J. M. Mitchison and M. M. Swann.

A close estimate of total dry mass of a homogeneous microscopic object can be obtained by measuring the refractive index difference (Davies and Wilkins, 1951; Barer, 1952). However, the heterogeneity of living cells makes obtaining total, integrated, dry mass values extraordinarily laborious and difficult.

Using the Smith interference microscope (manufactured by C. Baker, Ltd.), suitably modified, advantage can be taken of the optical integration of the field occurring at the objective back focal plane (BFP). Covering half the BFP with a suitable quarterwave plate in conjugate focus, a sensitive null point of identical intensities is achieveable. Twice the angular difference between successive readings, with a cell in the field (suitably "boxed" by adjustable leaves in conjugate focus with the image) and with the equivalent field alone, gives the integrated retardation of the entire cell.

By using ripple-free monochromatic illumination, the quarterwave plate in a rotating sector disk, and a photomultiplier and CRO, alternate intensities can be precisely balanced so that repeatable readings of ± 0.05° are obtainable. Experiments with models have confirmed the theoretical expectations and limitations of the method.

Preliminary data, obtained simultaneously, of volume and dry mass increases of an individual yeast (*Schizosaccharomyces pombe*) cell during growth and division, show that dry mass rises fairly regularly up to division, although some plateauing may occur. Volume increase stops prior to and during division but dry mass continues to rise. About 15 minutes following cleavage (27° C.), the combined dry mass of the daughter cells abruptly increases. Concentration is lowest just before division.

Damp chamber technique for rearing progeny from counted eggs of Mormoniella. David T. Ray.

The female wasp penetrates the puparium of the host blowfly, Sarcophaga, with her sting, paralyzes the pupa, feeds on the pupal juices in the space between the pupa and puparium. The wasp larvae develop to adults within the puparium, biting out a small opening to escape. In order to count the eggs they must be removed from the host puparium. These eggs were transferred to new host pupae which were inactivated by the sting of a female-sterile mutant type with oyster-white eyes. About 30 eggs were placed in a small hole which was made in each new host puparium. To avoid drying, which would prevent development from the counted eggs, the new hosts were placed in vials in chambers with saturated NaCl solution. A total of 5737 wasps matured from 7375 eggs of wild-type females, 78% survival. A hatchability rate of 95% has been found for wild-type Mormoniella eggs placed in Nujol. The difference between hatchability and survival is in part due to bad pupae and in part handling technique. The latter was remedied as the work progressed. Grouped according to percentages maturing in each vial the eggs showed 186/517 with less than 50%; 364/610, 60%; 415/621, 67%; 1278/1704, 75%; 1412/1644, 85%; 2082/2279, 91%. The high modal number indicates normally high viability of wild-type stock.

The importance of tissue mass in the differentiation process in Tubularia. Malcolm S. Steinberg.

When the hydranth is amputated from a tubularian stem, the tissue of the stem begins to move toward the amputation site, thus progressively increasing the cell population density at the distal end until a critical level is attained, at which time differentiation begins. Time-lapse motion pictures have been obtained which illustrate this process. If one prepares a large number of 10-mm. stems and then amputates the distal 1.5 mm. at different times during the distad movement of the tissue, the number of cells in the 1.5-mm. piece will be greater the longer the amputation of the piece is delayed. Similarly, if one amputates the proximal 1.5 mm. at different times, the number of cells it contains will be *fewer* the longer the amputation of the piece is delayed. If relatively few cells are present, differentiation will be considerably delayed, but eventually a tiny, complete hydranth will be formed at the distal end of the 1.5-mm. piece. In pieces containing more cells, differentiation will occur sooner and the regenerated hydranth will be larger. In pieces containing still more cells, differentiation occurs even sooner and the entire piece transforms into a hydranth. When this occurs, however, the degree of completeness of the hydranth becomes a function of the number of cells available, so that with fewer cells present the new hydranth may lack more or less of the proximal hydranth structures, while with more cells present a morphologically perfect hydranth tends to differentiate. Thus the time required for the differentiation process to be initiated bears an inverse relationship to the number of cells present, while the scale of organization of the regenerated hydranth bears a direct relationship to the number of cells present. The degree of morphological completeness of the regenerated hydranth also bears a direct relationship to the number of cells present, but only when the entire tissue of the piece transforms into a hydranth.

Responses of the diurnal melanophore rhythm of Uca pugnax to changes in temperature. G. C. STEPHENS.

The melanophores of *Uca pugnax* are normally expanded in the daytime and contracted at night. It is known that these alternations of condition continue in constant darkness for long periods of time and their twenty-four hour frequency remains intact at temperatures between 6° and 26° C. These reports have been verified in the present experiments. However, sudden changes in temperature within this range are effective stimuli in shifting the chromatophore rhythm.

If a group of animals which has been maintained in constant darkness at 18° C. is abruptly exposed to a temperature of 9.5° C. for a period of 12 hours and then returned to 18° C., the diurnal rhythm of the melanophores is set back by two to four hours. This process may be repeated after an additional 12 hours at 18° C. and produces an additional shift in the rhythm which sums with the first one produced. By alternating exposures to 18° C. and 9.5° C. it was possible to shift the rhythm 17 hours; five exposures to low temperature were involved.

The rhythm is not merely retarded during the sojourn at the lower temperature since control animals maintained at 9.5° C. for periods as long as 144 hours show a shift in diurnal rhythm of only two to four hours. When the animals remain at the low temperature for only three hours or six hours, a shift in timing is produced but smaller than that obtained with a twelve-hour exposure.

These shifts persist for at least ten days in constant darkness.

Electrical potential changes upon fertilization of the starfish egg. A. TYLER,[1] A. MONROY,[2] C. Y. KAO AND H. GRUNDFEST.[3]

Following the demonstration of a resting potential (40 to 60 millivolts) in the starfish egg (see above), the changes occurring in its value were determined by fertilizing eggs while impaled upon micro-electrodes. Within 15 to 30 seconds after insemination there is observed a drop (decreased negativity) of 5 to 10 millivolts during less than 5 seconds. During the subsequent 30 seconds the potential returns to the original value and within the next minute surpasses it by 10 to 15 millivolts where it remains steady. This was observed in each of six experiments in which successful fertilization was obtained. The initial drop corresponds closely with the time that the sperm are observed to reach the egg. The return and overshooting of

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[2] Fulbright Fellow.

[3] Supported by a grant from the Marine Biological Laboratory under its ONR Research Contract (Nonr-09703).

the original value correspond in time with the observed elevation of the fertilization membrane. If the larger potential observed after fertilization is not due simply to the formation of a better electrical seal of plasma membrane to the wall of the electrode, then it reflects changes in metabolic activity on the part of the egg. It may be related to the higher K value found (Monroy-Oddo and Esposito, 1951) in sea urchin eggs after fertilization. The temporary initial drop in potential suggests an increase in the rate of exchange of ions across the membrane during the first 5 seconds following contact of sperm with the egg.

Phosphate metabolism of starfish oocyte nucleoli. W. S. VINCENT.

The incorporation of P^{32} into various fractions of the starfish oocyte and nucleolus has been analyzed. Phosphate fractions were extracted by a modified Ogur procedure from whole homogenates of oocytes and isolated nucleoli after 2, 4, 6 and 8 hours of continuous exposure of ovaries to P^{32} in sea water. The relative specific activity of the P contained in cold acid-soluble organic P, hot alcohol ether-soluble P, hot acid-soluble P, and protein bound P was consistently higher in nucleoli than in whole egg fractions during the 2, 4, and 6 hour period. By approximately 8 hours all cytoplasmic fractions except the hot acid-soluble fraction were equal to the nucleolus. The hot acid-soluble fraction, identified as RNA, contained 13 times the S.A. of the cytoplasmic RNA at 6 hours, and at the later interval still 8 times that of the cytoplasmic RNA P.

Some implications of these results on interpreting nucleolar function will be discussed.

Ebony versus black eye color in Mormoniella. P. W. WHITING.

In addition to the expected dahlia and oyster-white sons of a compound female, *da/oy*-250.6, there occurred a single mutant with black eyes. This proved to be dahlia "ebony." Ebony, *eb*, resembles the previously reported black, *bk*, phenotypically but segregates independently and shows very different reactions with some of the other eye colors. All bright pigment appears to be removed by *bk* whether the colors are determined by the *R* series of alleles or by other factors. Thus scarlet black is white, vermilion black is light lavender (= dilute black with structural effect), dahlia black is dark lavender and the "red" pigment is taken out of wild-type brown and mahogany leaving black and dark gray, respectively. The gene *R* contains two elements designated *O* and *S*, either or both of which may mutate. Thus the mutant gene may be either singly or doubly recessive. The doubly recessive alleles such as orange, peach and tinged in combination either with *bk* or *eb* appear completely colorless resembling oyster, *oy*, a recessive in the *O* element. Dahlia, *da*, a single recessive in the *O* element, appears black in combination with ebony, *da eb*, in contrast to the dark lavender of dahlia black, *da bk*. This suggests that the *O*-element red of dahlia is changed to black by *eb*, but removed by *bk*. In contrast the *S*-element reds of mahogany, *mh*, vermilion, *vm*, scarlet-DR, *st*-DR, and scarlet-313, *st*-313, are unaffected by *eb*, so that these colors are similar to their respective combinations with *eb*. F_2 males from crosses of these *S*-element reds with ebony therefore occur in 2:1:1 ratio showing epistasis of these reds over ebony, but F_2 males from crosses of *S*-element reds with black show four colors in equal numbers.

On sex behaviour and sex determination in Crepidula fornicata. JAN Z. WILCZYNSKI.

I. The statistically significant sex ratios in chains of *Crepidula fornicata* are: 68% of males, 28% of females and 4% of the organisms in the transient state with reduced copulatory organs. Out of 235 disconnected (but free to reassociate) males, which survived being left for 10 months in a floating car in the harbour, 35% underwent the sex change into females, 5% were in the transient state, while 60% of males remained unchanged.

II. Two series of experiments of 4 and 6 weeks consisting in keeping the disconnected males either in 28–30° C. running water or in water at 0° C. changed daily brought no noticeable, statistically significant, induced sex changes. Nor did any color change of the gonads from orange (of male) to yellow (of female) occur.

III. The reciprocal injections of sea water extracts from males or females into females and males, respectively, resulted in very high mortality rate, but did not produce any sex changes in the few which survived.

IV. Cytological investigations of the "squashed" gonads taken from various sized organisms (smallest males to largest females) disclosed that they are hermaphrodites in every stage of their life cycle. The gamogonies of both sexes show a considerable polimorphic differentiation into, at least, three kinds of ovogonies and no less than 5 kinds of spermatozoids differing by the shape of their cells and nuclei or in their staining characteristics. The sex differentiated coloration (pink or yellowish with Neutral Red, violet-green or opal with methyl green) of ovogonies and spermatozoids has been obtained.

V. The conclusions are: (1) the aggregates of *Crepidula fornicata* are not the reproductive breeding associations but rather the hereditarily established feeding communities to procure and to utilize the stronger water current propelled by gills towards the mouths. The more so that (2) the sexes in the permanent chains are mechanically, by their distance, uncapable of any sexual intercourse, being, moreover, separated by one (or two) organisms sexually immature and inactive. The copulations, therefore, are being restricted to the smaller, the so-called additional or visiting, males. Probably, spawning females even do not need repeated insemination because their seminal receptacles are full of sperm. (3) The constant ratios in the natural as well as in experimentally induced conditions, as well as the orderly arrangement of the growing sizes in their chain formation, suggest that the sex determination in *Crepidula fornicata* is to be considered basically as genotypically controlled, representing the gradual increase of female factors which might be reduced to some exponential formula.

Tetrarhynch (cestode) life histories. R. T. Young.

There are three stages in the probable life history of a tetrarhynch, copepod (1st intermediate host), teleost (2nd intermediate host) and selachian, the final host.

The first of these steps was accomplished by Ruzkowski in 1934 with *Grillotia erinacea* and repeated by me at Woods Hole in 1953 in one experiment with another species (*Lacistorhynchus tenuis*). The third step I accomplished in 1949 with the teleost (Cymatogaster) and the shark (Triakis) in Southern California, and in one experiment only at Woods Hole in 1955 with the tetrarhynch (Grilotia) and the ray (Diaphanus). The second step, *i.e.*, transfer of the parasite from copepod to teleost, awaits accomplishment.

PAPERS READ BY TITLE

Membrane conductance and potential during normal and altered activity of squid giant axons. E. Amatniek, C-Y. Kao and H. Grundfest.[1]

The membrane conductance in a localized region of the squid giant axon has been measured simultaneously with the membrane potential in that region. Two glass-coated, 250 μ platinum wires have one end ground and polished to flatness. With the aid of a special manipulator the two opposing, axially aligned electrodes are brought to and pressed firmly against the sides of an axon at a site already impaled from above by a microelectrode or micropipette. When connected into a 31 kc Wheatstone bridge, as much as 12 to 15% change in resistive unbalance is observed during passage of a spike.

Increased conductance begins during the interval (about 20 μsec) when a spike of 118 mv attains 36 to 55 mv. Calculations indicate that recording of conductance change is localized to about 0.5 mm. from the center of the impedance electrodes. Peak conductance coincides with peak spike voltage, but the falling phase of conductance change outlasts that of voltage. Heightened conductance does not, however, persist throughout the hyperpolarisation phase of the spike. It ends earlier, as was inferred by Shanes, Grundfest and Freygang (1953) who found that approximately during the terminal 75% of the hyperpolarisation phase the membrane resistance increases (up to three-fold above the resting value; Amatniek *et al.*, to be published). Increased efflux of potassium therefore does not accompany all of this phase and cannot be responsible for its maintenance.

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[1] Supported by a grant from the Marine Biological Laboratory under its ONR Research Contract (Nonr—09703).

Spike conductance is a more sensitive indicator of membrane changes than is voltage. Massive injection of 0.3 M KCl may reduce the spike by 2 to 4 mv, while peak conductance falls as much as 50%. Variation of membrane potential therefore appears to be partly independent of ionic fluxes. Microinjected agents which depress electrical response decrease peak conductance even more sharply. However, persistence of some heightened conductance is a measure of persistence of graded activity at the injection site.

The effects of x-radiation on larval and pupal stages of the yellow mealworm, Tenebrio molitor Linn. Alan Priest Brockway.

In preliminary experiments, dosages of 4600 to 46,000 r were used on larvae of *Tenebrio molitor* Linn. Since the precise age of the larvae was unknown, specimens were divided into two weight groups as follows: Group 1 was comprised of larvae which fell into a weight range of 100.0–119.9 mg.; Group 2 contained larvae which weighed from 80.0–99.9 mg. In Group 1, when one-half of the control larvae had pupated, none of the irradiated larvae had pupated. In Group 2, none of the irradiated larvae had pupated when one-third of the control larvae had. It is believed that Group 1 which contained the heavier larvae contained specimens older than those in Group 2.

There was no observable difference in effects between doses ranging from 2750 to 4600 r when given to pupae within six hours after formation. At room temperature (approximately 24° C.) 7.5 days are required to complete pupation; the irradiated pupae, however, required 10.8 days. The irradiated pupae produced deformed adults. In general, not all of the cuticle was shed and the wings and elytra were undeveloped. In addition, what appeared to be islands of pigment were found on the elytra, legs and abdominal segments. Irradiation, therefore, inhibits the pupation of larvae and prevents the pupae from forming normal adults.

In determining LD 50 values, it appears that the lighter larvae which made up Group 2 are more radiosensitive than the heavier and presumably older larvae of Group 1. At 9200 r, the LD 50 of Group 1 was 18 days, that of Group 2 was 16 days; at 13,800 r, Group 1 was 22 days, that of Group 2 was 17 days; at 27,000 r, Group 1 was 11 days, Group 2 was 10 days; at 46,000 r, both groups at 8 days. Thirty larvae were used in determining each LD 50 value. Dosages greater than 46 kr were used only in Group 1 larvae with the following results: 69 kr gave an LD 100 in 7 days; 230 kr in 4 days; and 460 kr immediately.

A demonstration of the efficacy of muscular force in the opening of clams by the starfish, Asterias forbesi. Allison L. Burnett.

Many observations have shown that the valves of a quahog need not be opened widely to permit the insertion of the everted stomach by a starfish; a gape of 1 to 2 mm. suffices. The experiment described below indicates clearly that the starfish can produce such a small gape by muscular force alone, and that the action of toxic secretions on the adductor muscles need not be postulated. The hook of a small spring scale is inserted into a notch in the edge of the valve of a 2-inch quahog; the pull required to produce a very small gape is recorded. Both adductor muscles of the quahog are then severed, and the valves are wrapped with strong rubber bands sufficient to withstand a pull greater than that required to open the intact clam. In a typical instance, the clam was opened slightly by a pull of 4500 gms.; with the adductors cut, rubber bands requiring a pull of 5500 gms. were applied. A hungry starfish of 6-inch radius will accept this preparation, orienting the clam with the gape uppermost and assuming the familiar "humped" position. Within 5 minutes the valves have been opened to a distance of 2 mm.; they are held thus for about 3 minutes and then snap closed. Alternate opening and closing is repeated throughout a feeding period of about 1½ hours. After approximately 30 minutes the valves are held open as long as 10 minutes. Here, the starfish works against the tension of elastic bands not subject to fatigue or digestion; normally, the adductors probably progressively relax. The stomach of the starfish does not seem to be damaged by constriction between the closed valves, and it appears likely that in normal feeding the starfish may rest periodically, allowing the valves to close upon its everted stomach.

Further studies on the toxic factor in Phascolosoma.[1] ALFRED B. CHAET.

In 1951 the writer had established that, when *Phascolosoma gouldi* were immersed for 90 seconds in 76° C. sea water, death was brought about as a result of a toxic substance. This heat-stable, non-dialyzable toxin, present in the coelomic fluid of such scalded animals, proved lethal when injected into normal worms. Recent experiments have shown that the muscular tissue of the body wall is not the source of the toxic factor. The toxic factor is released or produced from the cellular portion of the coelomic fluid. No interaction between the coelomic fluid and the muscle is necessary in order to release the toxin from the cells of the coelomic fluid, since the factor is obtained by heating normal coelomic fluid *in vitro* (2 minutes at 76° C.). The substance was further isolated from samples of heated coelomic fluid, which had subsequently been both autoclaved (20 minutes at 15 lbs. per sq. inch) and dialyzed, by saturating these precipitate-free solutions with ammonium sulphate. This precipitated a fraction which proved to be toxic after the ammonium sulphate had been removed by dialysis. Preliminary analysis of this fraction indicates the presence of a carbohydrate other than a monosaccharide, as well as either protein and/or polypeptides. As yet further fractionation has been unsuccessful. In an attempt to find a semi-microbiological method for the detection of the toxic factor, *Arbacia punctulata* eggs were used as test objects. When these eggs are subjected to the toxin for from 2–6 minutes, the eggs fail to divide. Arbacia eggs are relatively sensitive to this material since as little as 6% of the toxin present in the coelomic fluid of heated worms is sufficient to be detected by this assay.

Purine and/or x-ray inhibition of Arbacia egg development. RALPH H. CHENEY.

The effects of 1:3:7 CH_3 2:6 dioxypurine, caffeine, stated in 1948 by Cheney, and the mitotic inhibition in prophase by this compound and by x-radiation reported in 1954 by Cheney and Rugh, are compared with the effects of the parent molecule, pure purine-in-filtered-sea-water (PSW), in a series of molarities combined with and without x-radiation (30,000 r). Critical inhibitor effects occur primarily at the amphiaster and at blastula formation. Fertilizing ability of sperm was unaffected by the x-ray dosage employed (30,000 r). This fact was established by direct x-radiation of wet sperm and their use subsequently for fertilization. Controls were compared with the results of the following treatments: (1) inseminating normal eggs and sperm in previously filtered, irradiated SW and observing the fertilization process and development to pluteus; (2) eggs irradiated in SW × normal sperm with development in irradiated SW; (3) eggs mixed with sperm and developed in non-irradiated, specific molarities of PSW; (4) purine-treated eggs × sperm with development in irradiated SW; (5) purine-treated eggs × sperm with development in irradiated PSW.

The evidence substantiates the previous suggestion that the inhibitory (and, in high concentration, the complete arresting) effect of the trimethylated dioxypurine, or of heavy dosage x-radiation, is initiated via different mechanisms. This assumption is based upon the fact that pure purine itself affects similarly the mitotic behavior primarily in the prophase, as does the x-radiation, but there is no significant evidence of synergistic, summation nor antagonistic action between the chemical and physical agents.

Dinitrocresol and glucose-C^{14} utilization in Arbacia eggs. G. H. A. CLOWES, A. K. KELTCH, C. P. WALTERS AND M. E. KRAHL.

Dinitrocresol (DNC) stimulates oxygen consumption and blocks cleavage in Arbacia eggs; it inhibits oxidative phosphorylation and glucose-6-phosphate dehydrogenase in egg extracts. DNC effects on metabolism of glucose-1-C^{14} (g1), glucose-2-C^{14} (g2), and glucose-6-C^{14} (g6) have been studied by the technique given by Krahl *et al.* in a previous abstract; two concentrations were used, 8×10^{-6} M, which stimulates respiration optimally and inhibits cleavage 50 per cent, and 1.28×10^{-4} M, which brings respiration down to normal and inhibits cleavage completely. Representative total corrected c.p.m. collected as $BaCO_3$ were: unfertilized, with O, 8×10^{-6}, and 1.28×10^{-4} M DNC, respectively, from g1, 5900, 11600, 6600; from g2, 1900, 12300, 4700; from g6, 340, 9300, 1560. Corresponding values for eggs fertilized 25 minutes were: from g1, 3400, 4800, 4500; from g2, 2000, 4400, 4000; from g6, 565, 3200, 2100. The values for plutei were: from g1, 39600, 51800, 63800; from g2, 19400, 43000, 67500; from g6, 11900, 39500, 62100. DNC produced only a minor increase, if any, in uptake of labeled glucose from the medium. One effect is therefore to increase CO_2 production from glucose, particularly by a pathway favoring conversion of carbon 6 to CO_2. Representative total c.p.m. collected in other fractions after incubation of unfertilized eggs with O and 1.28×10^{-4} M DNC were, respectively: for NPL fraction from g1, 1380 and 60; from g2, 1920 and 60; from g6, 1600 and 50; for fraction I from g1, 3400 and 2850; from g2, 5900 and 3100; from g6, 4750 and 2800; for fraction II from g1, 14100 and 3800; from g2, 13500 and 5300; from g6, 12600 and 4600. DNC therefore reduced the incorporation of glucose carbons into the nucleoprotein fraction almost to zero and also that into the barium fraction insoluble in alcohol (II). These fractions are being studied further.

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[1] This investigation was supported in part by Grants No. R-625-AL and R-625-AK, Coe Research Fund, Univ. of Maine.

Concerning the spermatozoon and fertilization in the egg of Sabellaria vulgaris. ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

In the presence of eggs, egg water, dilute solutions of ammonium hydroxide and apparently to some extent upon contact with the glass of a slide or cover-slip, the spermatozoon of *Sabellaria* gives rise to a projection from the acrosome region. This acrosome filament varies from a short thick rod to a delicate thread which is sometimes longer than the length of the head and middle-piece together. To initiate fertilization the acrosome filament apparently makes contact either with the surface of the egg proper, or with one of the many long and sometimes branching filaments which already extend from the surface of the egg to the unevenly elevated vitelline membrane. There is some evidence that the acrosome filament then passes into the protoplasm of the egg as the first element of the spermatozoon of which it is an integral part.

Some factors related to sperm entry in two species of Asterias. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

Asterias forbesii. When sperm is added to sea water containing eggs, some of the spermatozoa come to appear as follows: the head and middle piece are each much rounded in outline and the flagellum arises from a point at or very near their junction; from the acrosomal region of the head there projects a very thin but rather rigid filament measuring sometimes as much as 24 microns, and of the order of one fourth to one third the length of the flagellum. This acrosome filament is the first part of the spermatozoon to make contact with the surface of the egg, stimulating the egg's fertilization reaction. There is some, but not conclusive, evidence that the acrosome filament then moves on into the protoplasm of the egg, still acting as an integral part of the spermatozoon. The position of the acrosome filament with reference to the egg surface may be either radial or oblique, and the main body of the spermatozoon subsequently moves toward the egg along the same path. Seemingly identical acrosome filaments are found in spermatozoa which approach and successfully enter eggs, in spermatozoa which approach but fail to enter the eggs subsequently and in spermatozoa near but not even directed toward any specific egg.

In *Asterias vulgaris* spermatozoa added to eggs in sea water show the same structural relationships as seen in *A. forbesii.* The long rather rigid acrosome filament of the spermatozoon may occupy either a radial or an oblique position with reference to the egg surface. The spermatozoon can possess the acrosome filament even when not attached to a specific egg.

The spermatozoon and sperm entry in the egg of the holothurian, Thyone briareus. LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

Reacted spermatozoa are found occasionally in suspensions of spermatozoa in sea water, in proportions of approximately 20% in suspensions of spermatozoa in egg water, and in higher proportions in suspensions of spermatozoa in dilute solutions of ammonium hydroxide in sea water. Many are also found close to or attached to eggs following insemination. In appearance the reacted spermatozoon has the middle piece and head each much rounded in outline and the flagellum arises from a point at or very near their junction. The exceedingly tenuous filament which projects from the acrosome region is usually 35–50 microns in length with occasional specimens as long as 90 microns and is of the order of half to nearly the full length of the flagellum. Successful sperm penetration, and polyspermy, occurred very slowly in germinal vesicle eggs eviscerated artificially from the gonads. In response to the stimulus of the spermatozoon these eggs often produced cones which were broad at the base but which crept outward, surrounding the acrosome filament in a narrow sleeve of protoplasm so fine as to be considered almost filamentous itself. However, the acrosome filament could be seen within such hyaline cones. Successful fertilization in normally shed eggs followed the same pattern but occurred much more rapidly.

The antimitotic activity of thiocyanate extracts of puffer ovaries. PIERRE COUILLARD.[1]

Crude extracts of the ovary of the fish *Spheroides maculatus*, the common puffer, have been shown to inhibit the first cleavage of marine eggs and to exert an anti-thrombic action on the clotting of mammalian blood. The active agent appears to be a protein-bound carbohydrate, probably a heparin.

An attempt was made to isolate this complex using the thiocyanate procedure successfully employed by Snellman *et al.* on mast cell material. Extraction of whole ovaries by molar potassium thiocyanate followed by exhaustive distilled water dialysis and lyophilization of the extract, yields a dry preparation amounting to 10% or so of the wet starting material. This product inhibits cleavage of the *Chaetopterus* egg; it shows activity in dilutions as low as 1 to 5000, in spite of the fact that only a small part of it goes into solution in sea water. The active agent is thermolabile and unaffected by thorough extraction by fat solvents. From sea water solutions, the bulk of the material as well as most of the activity precipitates out between 50 and 60% saturation with ammonium sulfate and between 0 and 25% alcohol in the cold.

The product contains carbohydrates: the equivalent of 0.05% glucose (by the anthrone test), of at least 1.5% glucuronolactone (by the Dische carbazole method) and of 3 to 3.5% hexosamine.

Attempts to inactivate the complex by digesting the protein with chymotrypsin or by oxidizing the carbohydrate with periodate have not succeeded so far; the effect of the complex on blood coagulation was not studied but the possibility is still strong that this thiocyanate-soluble fraction contains the heparin-protein complex responsible for the antimitotic and anticoagulant activity of crude puffer ovary extracts.

The induction of haploidy in Tetrahymena pyriformis following x-irradiation. ALFRED M. ELLIOTT AND GORDON M. CLARK.[2]

Since *T. pyriformis* is normally diploid, the task of determining the presence of recessive mutant genes requires two generations, whereas if autogamy, cytogamy, or haploid strains could be induced, this information could be acquired in one generation. Exhaustive efforts to induce autogamy in variety 1 have failed, hence the other two possibilities were investigated.

An effort was made to induce cytogamy by separating the two conjugants during various stages of meiosis and before pronuclear interchange. The use of detergents, proteolytic enzymes, electric and heat shock, all failed to separate the animals. Vigorous mechanical agitation, however, did accomplish the separation but the "exconjugants," instead of continuing on in meiosis as was hoped, either remained in the stage they had reached when separated or became amicronucleate. Approximately 40% of the cells lost their micronuclei and many of these mated either with normal or other amicronucleate animals. In any case, none of the separated cells survived. Apparently the process of separation inflicted irreparable damage to the cells.

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[1] Holder of a Special Scholarship, National Research Council of Canada.

[2] This investigation was supported in part by a research grant (PHS G3588) from the National Institutes of Health, Public Health Service.

Efforts to produce animals with the haploid number of chromosomes were more fruitful. Mating type II of variety 2 was exposed to sublethal doses of x-rays (10,000, 50,0000, 100,000, 300,000, 400,000 r). The cells were incubated in broth for 24 hours following irradiation; then 90 clones were established, 18 from each of the five radiation levels. Each clone was then mated with unirradiated cells. Meiosis was normal in all clones from the lower levels of radiation, but abnormalities appeared in those matings where one cell had had 300,000 r or more. One clone from the 400,000 r series showed radical micronuclear behavior: meiosis was so abnormal the third prezygotic division seldom began and was never completed. The haploid migratory pronucleus from the normal mate crossed to the irradiated cell and there established the full nuclear complement of two micronuclei and two macronuclear anlage. Proof that these cells were haploid was established when exconjugant clones from this cross were mated to normal cells, and five univalent chromosomes were counted during the first meiotic division. Erratic distribution of the chromosomes in anaphase occurred, the spindle usually containing two univalent chromosomes at one pole and three at the other, or some other distribution of the five univalents. Such haploids should be a useful tool in genetic analyses.

Thermal denaturation of lobster hemocyanin. JOSEPH H. FLEISHER AND MAX A. LAUFFER.

Lobster hemocyanin was purified by high speed centrifugation in the manner described by Lauffer and Swaby in 1955. The pellets were extracted with 0.02 *M* acetate buffer. Separate portions of the extract were adjusted to pH 7.40 with 0.05 *M* phosphate, to pH 8.48 with 0.05 *M* trihydroxy amino methane, and to pH 9.16 with 0.05 *M* glycine-NaOH. Ionic strength was maintained at 0.13 by the addition of appropriate quantities of NaCl.

The rate of denaturation was studied over the range of 58° C. to 73.6° C. The concentration of protein remaining in solution after known times of incubation at a given temperature was determined by means of a refractometer. Denaturation followed first order kinetics. At pH 7.4, rate constants of 0.05, 0.24, 0.52 and 1.8 min^{-1} were obtained at 65.6, 69.6, 71.5 and 73.6° C., respectively. At pH 8.48, rate constants of 0.10, 0.22, 0.51 and 1.2 were obtained at 63.0, 64.8, 66.9 and 68.8° C., respectively. At pH 9.16, rate constants of 0.02, 0.06, 0.15 and 0.32 were obtained at 58.0, 60.0, 62.0 and 64.0° C., respectively.

The rate constants were plotted against the reciprocals of the absolute temperatures. From the slopes of the curves obtained at each pH, the activation energy was found to be 9×10^4 cal/mol. Extrapolation of the same graphs permitted a comparison of $\log_{10}$ k with pH at constant temperature. At both 65 and 66° C., $d \log_{10} k/d\text{pH}$ was $+0.9$, probably not significantly different from $+1$, over the pH range studied.

X-ray induced cessation of gamete production by adult female Artemia. DANIEL S. GROSCH AND ROBERT L. SULLIVAN.

Further experiments now verify the sterilizing dose of x-rays for the brine shrimp *Artemia salina* (American strain) published earlier in this same journal. The suspected range was covered by delivering 2000, 2250, 2500, 2750 and 3000 roentgens. Twenty adult females were exposed at each dose. Thus with an equal number of controls, a total of 120 females was considered. After treatment, each female was paired with an untreated male and subsequent gamete deposit determined until death. Only at 2250 r or below may the deposit of more than one brood be expected, and at this and lower doses, no greater time elapses than the typical two to three days between successive broods. Therefore no temporary sterility has been demonstrated. Since a full ovisac is our criterion of female maturity, the single brood deposited at all doses comprise those gametes formed previous to treatment. The threshold for cessation of gamete production in female Artemia is hereby placed at 2250 r (delivered in 54 seconds).

Loss of the micronucleus and reduction in the number of kineties in a hymenostome ciliate.[1] George G. Holz, Jr.

A hymenostome ciliate, differing in but one respect (possession of a single caudal cilium) from members of the genus *Tetrahymena*, was isolated from pond water and established in monoxenic culture, in Cerophyl infusion, with *Aerobacter aerogenes*. A single ciliate from this clone was washed free of bacteria to establish an axenic culture in a trypticase-brewer's yeast-Cerophyl medium. One month after isolation, the axenic clone was uniformly amicronucleate, while the monoxenic clone was uniformly unimicronucleate. The pattern of infraciliature of the two clones was identical, with one exception; the amicronucleate clone had 17–21 primary ciliary meridians, the unimicronucleate clone had 22–25. The nuclear constitution and infraciliature of these two clones have remained stable to date (one year). Other axenic clones established from the monoxenic clone have remained unimicronucleate, and monoxenic clones established from the original axenic clone have remained amicronucleate to date (8 months).

It is considered that (1) the reduction in the number of ciliary meridians was related directly or indirectly to the loss of the micronucleus, or (2) the changes occurred independently and hence were unrelated. From the first alternative it would follow that maintenance of the characteristic number of kineties is dependent upon the presence of the micronucleus. Current view of the micronucleus among protozoologists assigns the structure a purely generative function. The second alternative requires that the two events were fortuitously coincident within the month between isolation and examination. Loss of the micronucleus in other ciliates has been explained as resulting from degeneration, or aberrations of nuclear reorganization and fission. Permanent loss of kineties might result from mutation of kinetosomes or alteration of their cytoplasmic environment by mutation of a nuclear genetic determinant. The mechanism by which progeny of amicronucleate individuals with a reduced number of kineties come to dominate a clonal population is at present under study.

A field character for distinguishing Palaemonetes vulgaris from Palaemonetes pugio.[2] Charles E. Jenner.

The revision of the Palemoninae by Holthuis (1952) shows that the specific identity of the much-studied *Palaemonetes* of the Woods Hole region is in doubt. At least two species are known to occur in this area—*Placmonetes vulgaris* and *Palaemonetes pugio*. Holthuis separates these species by the length of the carpus (longer than palm in *P. pugio*, about equal to or shorter than palm in *P. vulgaris*), shape of the rostral point (dagger-like in *P. pugio*, dorsal teeth up to tip in *P. vulgaris*), number of dorsal rostral teeth behind the orbit (one in *P. pugio*, two in *P. vulgaris*), and other structural features. With experience these characters can be observed in the field, but a more obvious one is presented by the color of the eyestalks. Variations between individuals occur, but, in general, the eyestalks of *P. pugio* are much more yellow than those of *P. vulgaris*, the latter being more red-brown in color with little yellow pigment. Shrimp with decidedly yellow eyestalks, upon more careful check, have always been found to be *P. pugio*.

The two species differ in local distribution but are frequently found together. *P. vulgaris* apparently has a wider range locally than *P. pugio*, both horizontally and vertically in estuaries, *P. vulgaris* being found in more saline and deeper water than *P. pugio*. Both species seem to stop their reproductive activity during the last of August or early September.

Several hundred shrimp have been collected near the M. B. L. dock and wall behind the brick laboratory, and only *P. vulgaris* has been found. Since this is apparently the principal source of *Palaemonetes* for the Supply Department, it can probably be assumed that most of the experimental work on *Palaemonetes* at the Marine Biological Laboratory has been correctly referred to *P. vulgaris*.

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[1] This investigation was supported in part by a research grant (E-797) from the National Microbiological Institute of the N.I.H., P.H.S.

[2] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C3).

The eyestalk color should allow experimentalists to check quickly the species identity of their *Palaemonetes*; however, a third species, *Palaemonetes intermedius,* probably occurs in this area also (Holthuis, 1952).

Changing electrical constants of the Fundulus egg surface. C. Y. Kao.

The resistance and capacitance of the plasma membrane of the Fundulus egg have been determined by means of two intracellular electrodes, one for polarizing and one for recording against an external ground electrode. Measurements of the current-voltage relationship and the distortion of an applied square pulse yield values of membrane resistance and capacitance, respectively. In the unactivated egg membrane resistance averaged 3450 ohm-cm^2 with a range of 1860 to 5750 ohm-cm^2. Upon activation, membrane resistance increases steadily till the end of one hour and thence remains at a stable level averaging 13290 ohm-cm^2, with a range of 8870 to 25000 ohm-cm^2. For any one egg, the increase is consistent from 2- to 7-fold. When an activated egg is immersed in hypertonic (0.2 to 0.4 M) sucrose solutions in sea water, membrane resistance drops rapidly within seconds towards the value present in the unactivated egg. This decrease of membrane resistance accompanies the swelling of the egg proper previously described. While remaining in the sucrose solution, membrane resistance returns, much faster than volume change, to the level of the activated egg. During the changes occurring in activation or when immersed in sucrose solution, membrane capacity remains constant within experimental errors at an average of 0.5 μF/cm^2. The results suggest that the alterations in the plasma membrane of the Fundulus egg upon activation, which ultimately results in the sparing permeance to ions, is due to changes in the resistive components, probably the effective pore size.

No consistent significant membrane potential has been obtained in either unactivated or activated eggs. This point, however, should not be emphasized until the cation contents of the egg have been analyzed.

Pressure-volume relationship in the Fundulus egg. C. Y. Kao.

Previous work (Kao, Chambers and Chambers, 1954; Kao and Chambers, 1954) showed the existence of an internal hydrostatic pressure within the activated or fertilized Fundulus egg which is surrounded by an inelastic chorion with a constant volume. The pressure, which is attributable to a colloidal material in the perivitelline space, can be reversibly abolished by immersing eggs in hypertonic solutions of non-electrolytes. As shown previously, the internal hydrostatic pressure reaches a steady state of 150 mm. Hg above atmospheric pressure in a fully activated egg. The volume of the egg proper in this state attains a minimum. When hypertonic solutions of mannitol or sucrose made up in sea water are placed external to the whole eggs, the pressure within falls to atmospheric level or less; the egg proper swells to take up most of the perivitelline space. After periods of time varying from 5 to 30 minutes, the pressure increases again and is accompanied by a second shrinking of the egg proper. For any one egg, the product of the internal hydrostatic pressure and the volume of the egg proper, PV, remains constant in the following conditions: (1) in the fully activated state, (2) at the minimum pressure level when the egg proper is fully enlarged in hypertonic sucrose solutions, and (3) during the return of the internal hydrostatic pressure and accompanying shrinking of the egg proper while remaining in sucrose solutions. The variation of PV in these conditions is less than 2% and indicates a close relationship between the perivitelline pressure and the volume of the egg proper. This relationship, however, is only a manifestation of the dependence of the egg proper on the state of the perivitelline colloid to maintain its sparing permeance to water and ions.

Further investigation on hematochrome accumulation in the alga Phycopeltis hawaiiensis. John W. King.

The accumulation of hematochrome (a combination of carotinoid pigments) in species of the genus *Phycopeltis* has been described previously by the writer and other investigators who observed that this pigment accumulation depends upon environmental conditions and physiological activity of cells. *Phycopeltis hawaiiensis* is an algal epiphyte that grows normally on the leaves of pharenogams in Hawaii but has been found to grow also on mineral agar. The purpose of this investigation is to determine what effect certain light conditions and x-radiation have on the accumulation of hematochrome in this alga.

Media containing Knop's solution in 2 per cent agar were inoculated with *P. hawaiiensis* and grown for 15 days in diffused light. One half of these cultures was exposed to x-rays for ten minutes at a dose rate of 60 roentgens per minute. Both classes were grown for 30 additional days in natural daylight or fluorescent yellow light or fluorescent green light. Plants from cultures were then dried, weighed, and extracted of carotinoids with petroleum ether. To measure the amount of carotin present, a colorimeter was used.

The x-radiated natural daylight-grown plants contained 2 per cent more grams carotin per liter per milligram of dry plant than did the non x-radiated plants grown in natural daylight. The x-radiated fluorescent yellow light-grown plants contained 81 per cent more grams carotin per liter per milligram of dry plant than did the x-radiated fluorescent green light-grown plants. These results suggest that hematochrome accumulation in this alga depends upon the quality of light reaching the cells. Although the difference in carotinoid content of the x-radiated and non-x-radiated was not found to be very great, the effect this energy has upon hematochrome accumulation cannot be overlooked.

The nature of the reflecting pigment in the arthropod eye. L. H. KLEINHOLZ.

The components of the retinal reflecting pigments of Homarus and Limulus were studied. In Homarus separation by paper partition chromatography, followed by inspection in ultraviolet light, and subsequent visualization of purines by forming the mercuric sulfide complex, revealed three and sometimes four spots. Two of the spots were purines, one of which had previously been identified as uric acid; the second purine spot did not appear consistently, was usually faint in appearance and may therefore be present in low concentration; the other two spots were fluorescent in ultraviolet light. R_f indices of one of the fluorescent spots in a series of different solvents, as well as the properties of solutions of this fluorescent material eluted from the excised areas of the paper, indicated that the substance was xanthopterin. Absorption spectra of this eluate in 0.1 N HCl showed absorption maxima at 231 mμ, 261 mμ, 355 mμ, compared with absorption maxima of standard xanthopterin solution at 230 mμ, 259 mμ, 355 mμ. Absorption characteristics of the second fluorescent spot have not yet been determined. Reflecting pigment in Limulus is found in the retinas of the large compound lateral eyes, as a major component of the so-called rudimentary eye located in the postero-medial aspect of each compound eye, in the median eyes, and in deposits near the median eyes somewhat similar to the "rudimentary eyes." A single spot with the properties of a purine was revealed by using a variety of solvents for partitioning on paper. Comparison of the R_f indices of spots of the reflecting pigments, of standard purine solutions, and of combined spots of pigment and standard purines showed that all these reflecting pigments of Limulus were guanine.

Pathways of glucose-C^{14} utilization in Arbacia eggs. M. E. KRAHL, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

Extending previous work by the authors directed toward elucidating carbohydrate metabolism in Arbacia eggs and its importance for energy supply and synthesis of nucleic acids, Arbacia eggs or embryos were incubated 2 hours at 20° C. in 4 ml. sea water containing 600,000 counts per min. of glucose-1-C^{14} (g1), glucose-2-C^{14} (g2), or glucose-6-C^{14} (g6), each at total concentration of 0.0006 *M*. Three aliquots (each 80 mg. wet wt.) of the same batch of eggs were run concurrently with the three sugars. Total corrected c.p.m. were determined on gas counter for respiratory CO_2 (as $BaCO_3$), for the acetone-ether dried, acid-insoluble fraction (NPL, containing nucleoproteins, some lipid), for the barium fraction insoluble in H_2O (I), and for the barium fraction insoluble in 80% alcohol (II). Representative total c.p.m. collected as $BaCO_3$ were: unfertilized, from g1, 4375; g2, 1345; g6, 260; fertilized 25 minutes, from g1, 3355; g2, 1680; g6, 350; 24 hr. plutei, from g1, 52200; g2, 24100; g6, 17900. For other fractions the total c.p.m. recovered did not differ substantially with the labeling of glucose; average corrected values were: unfertilized, NPL, 600; I, 3800; II, 4800; fertilized, NPL, 400; I, 1350; II, 3640; plutei, NPL, 12100; I, 27000; II, 7500. The fractions are being analyzed chromatographically. Provisional conclusions are: first, in unfertilized or recently fertilized Arbacia eggs, glucose is used principally via the Warburg-Dickens TPN shunt, as previously postulated by the authors in 1954 from measurements on enzyme content of Arbacia; in plutei a larger fraction of glucose is used via the glycolytic pathway; second, synthesis of nucleoprotein from glucose is very slow in unfertilized or recently fertilized eggs, but is 20–40 times as fast in the plutei.

How sea stars open bivalves. Marcel Lavoie and George G. Holz, Jr.

The toxic secretion and force theories of the mechanism by which sea stars open bivalves have been re-examined critically.

Aqueous extracts prepared by a variety of methods from the organs of inactive and feeding *Asterias forbesi* were introduced into the mantle cavity and the adductor muscle of *Mytilus edulis.* Each mussel was subjected to a pull of 800 grams for 45 minutes. Data from 1000 tests indicate that such extracts do not influence the rate of shell opening more than similar injections of sea water.

The force exerted by the tube feet during predation was measured directly by substituting a steel coil spring for the adductor muscle of living *M. edulis.* A manometer connected to the spring revealed the application of forces in excess of 3000 grams during feeding. *A. forbesi* can insert its eversible stomach into the shell within 3 minutes after the pull begins and via an opening as narrow as 0.1 mm. The sea star's body does not move during this process so that the force needed for the pull is believed to be derived from the musculature of the tube feet.

These experiments are believed to provide proof for the force theory which heretofore has been accepted on the basis of experiments which measured only the adhesive power of the tube feet.

Inhibition of metamorphosis of tadpoles of the tunicate, Amaroecium constellatum. William F. Lynch.

Observations were made on newly-hatched tadpoles by using the following inhibitors of bryozoan metamorphosis: cold sea water (5–9° C.), a mixture of 80 cc. sea water/20 cc. of isotonic $MgCl_2$, Moore's Ca-free medium, a 0.1% solution of chloral hydrate in sea water, and a 3.3% crude sea water solution of minced ovarian tissue of the puffer, an anti-mitotic agent contributed by Dr. Pierre Couillard. The pH of all these media was adjusted to 7.9–8.0 by NaOH. Cold sea water, which immobilized the tadpoles, was more effective than the other agents, giving 95% inhibition during four-hour periods of observation. Other larvae, inhibited for twelve hours at 5° C., metamorphosed normally. Inhibition was reversible and swimming began at about 10° C. The sea water-$MgCl_2$ mixtures allowed only 28.7% metamorphosis, as opposed to 76.0% of the controls, the t ratio being significant at the 1% level. Inhibition was reversible within the two hour periods of observation. Tadpoles washed three times in cold Ca-free sea water and transferred to a fourth dish of the same medium at room temperature showed about 44% inhibition. Ca-free sea water, while not preventing metamorphosis in all the tadpoles, as it does in *Buguta* larvae, interfered with the mechanism of attachment and allowed only 3% affixation. Unattached larvae, almost invariably geonegative, did not complete the metamorphic process. The extract of ovarian tissue gave approximately 60% inhibition without killing the larvae. Tadpoles, kept for 1½ hours in cold chloral hydrate solution, showed a slight inhibition when the medium reached room temperature. A 1% and a 2% solution of heparin in sea water, contributed by Miss Muriel Lippman, gave no inhibition of metamorphosis in either ascidian or bryozoan larvae.

Sterols in the body fluids of marine worms. Sr. Elizabeth Seton MacDonald and Charles G. Wilber.

In general there is no wealth of information about the organic constituents in the body fluids of invertebrates. Analyses were made of the total sterols in the body fluids of various marine worms from the Woods Hole area. The results in mg. sterol per 100 ml. of body fluid, to date, follow: *Amphitrite* whole fluid (w.f.), 253; cell free fluid (c.f.), 11; *Cystenides* w.f., 30–100; c.f., 9; *Glycera* w.f., 234; *Nereis* w.f., 60. Specimens of *Amphitrite* were kept in running sea water in the laboratory for 9 days after which time the average sterol content of the body fluid was: w.f., 121 and c.f. 14 mg. per 100 ml. It is known that the amount of glucose in the coelomic fluid from *Phascolosoma* changes after exposure to elevated temperature. Does the sterol content also change? Control values at 23° C. were 5.2 mg. sterol per 100 ml.; after exposure to 31° C. for one hour, 3.3; after exposure to 36° C. for one hour, 12.0. In *Glycera* the sterol content of whole body fluid dropped from 234 to 217 mg. per 100 ml. after heating specimens for one hour at 36° C. It is evident that the sterol content of the body fluids in marine worms fluctuates with temperature and with the time during which the animals are kept in the laboratory.

The energetics of cell division: fluctuations in the structural state of the cortical plasmagel layer of the Arbacia egg in relation to the first and second cleavage divisions. DOUGLAS MARSLAND[1] AND ARTHUR ZIMMERMAN.

Pressure-centrifuge measurements of the displacement of the red pigment bodies present in the strongly gelated cortical cytoplasm (plasmagel layer) of the fertilized Arbacia egg were made at one-minute intervals between insemination and the completion of second cleavage. In all the experiments, a constant temperature of 20° C. was maintained. Thus the periods—between insemination and first cleavage (50% furrows) and between the first and second cleavages—remained quite constant at 58 (± 2) minutes and 33 (± 1) minutes, respectively. In order to achieve a reasonably rapid displacement of the pigment bodies it was necessary to use a rather high centrifugal force (41,000 × gravity) and a high pressure (8000 lbs./in.^{2}). The treated eggs were not injured appreciably, however, since they gave rise to normal plutei.

The experiments show that fertilization initiates a rather complex series of large, rapid changes in the gelational state of the cortical cytoplasm. Within 13–14 minutes following insemination the plasmagel layer, which is quite weak in the unfertilized egg, displays an enormous increase (at least forty-fold) in its structural strength. Now it requires about 160 seconds of centrifuging to displace all of the pigment bodies from the centripetal hemisphere, as compared to less than 4 seconds in the unfertilized egg. This initial rise is followed immediately by: 1) a quick drop (to about 130 at 17–18 minutes); 2) a second sharp rise (to 170 at 22–23 minutes); 3) a second quick drop (140 at 27–28 minutes); and 4) a third sharp rise (180 at 33–34 minutes). Then the high level is maintained until first cleavage is achieved, whereupon, within two minutes, the gel strength falls off rapidly to about 100.

Similar changes occur during the second division cycle, except that only two high points are displayed, the first at 9–10 minutes after first cleavage and the second, 3–4 minutes before second cleavage, with an intervening load level which endures from the 15th to the 25th minute of the 2nd cycle.

Presence in the extracts of sea urchin ovaries of a factor that accelerates cleavage.[2] VALY MENKIN.

The ovaries of *Arbacia punctulata* were homogenized with the addition of several ml. of distilled water. The whole extract *per se* induced variable effects on the appearance of cleavage when Arbacia ova were exposed to it for 20–30 minutes prior to fertilization. The aqueous extract was then centrifuged at about 4000 R.P.M. in a Servall angle centrifuge for approximately 10 minutes. One ml. of the supernatant added to ova in 9 ml. of sea water induced definite acceleration of cleavage. In 7 experiments in an average of 44.1 minutes, the experimental ova averaged 32.6% in the first cleavage as against 15.4% in the controls. In the second or subsequent cleavages in 56 minutes, the experimental averaged 22.3% and the controls 8.6%. About 4 to 5 ml. of this supernatant fraction was then dialyzed in the cold at about 10° C. for several days against 20 ml. of distilled water. The diffusate, a clear yellowish fluid, was found definitely effective in accelerating the onset of cleavage in concentrations ranging from 0.5 ml. to 1 ml. per 9.5 or 9 ml. of sea water. In 18 experiments, in 49.2 minutes the experimental averaged 39.3%

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in the first cleavage as against 20.3% in the controls. In the second cleavage or over in 61.9 minutes the number of experimental ova averaged 27.2% and the controls 4.7%. The accelerator factor in the diffusate was thermostable, withstanding boiling for 30 minutes. Similar effects were obtained if the diffusate was added several minutes following fertilization. Absorption spectrum with the aid of Drs. J. S. Roth and M. Levy indicated a peak around 265 to 270 millimicra. The diffusibility, thermostability, and the absorption spectrum suggest that the accelerator factor may well be a nucleotide.

Presence in the extracts of sea urchin ovaries of a factor that retards cleavage.[1] VALY MENKIN.

Investigators have demonstrated the presence of an antimitotic factor in the ovaries of the starfish (Heilbrunn *et al.,* 1951, 1954). The whole aqueous extract obtained from the ovaries of *Arbacia punctulata* induces either a slight acceleration or a retarding effect on the cleavage development of the fertilized ova. Two opposed factors appear to be present in the extract. Observations have already been described on the presence of an accelerating cleavage factor in the supernatant fraction and in its diffusible portion following dialysis. The indiffusible part yields inconstant results. It is, in contrast to the diffusible fraction, Biuret and Ninhydrin positive $(NH_4)SO_4$ precipitation at one-third saturation has failed to yield an active fraction. In 11 experiments with the indiffusible fraction, a slight retarding effect in the onset of cleavage has been observed in the second or subsequent cleavages, the experimental averaging 9.3% and the controls 20.5%. There apparently is some degree of thermostability; but boiling has at times also induced a reversibility of effects. Absorption spectrum measurements reveal a peak in the range of 265 to 270 millimicra. The data are not inconsistent with the possibility that there may be a nucleoprotein involved. The principal retarding factor is, however, recovered with the residual or sedimented fraction following centrifugation of the original extract. In 13 experiments, in 46.8 minutes, the number of experimental ova averaged 10.3% in the first cleavage, and the controls 40.6%. In 63.8 minutes, the experimental averaged 19.1% in the second or subsequent cleavages, and the controls averaged 39.1%. Plutei development has occasionally been observed with the accelerator factor, but never with the retarding factor.

The action of some metal ions and metal chelating agents on the motility and respiration of starfish sperm.[2] CHARLES B. METZ AND CARL W. BIRKY, JR.

Chelating agents enhance the motility of aged sea urchin sperm and extend the life span of this sperm (Tyler and Rothschild, 1951). These effects involve no appreciable increase in rate of oxygen consumption but appear to depend largely upon anaerobic metabolism. Apparently the effects depend upon removal of metal ions.

In view of these results it appeared of interest to examine starfish sperm for similar effects. Starfish sperm (*Asterias forbesii*) in 1–5% sea water suspension are ordinarily virtually immobile but addition of a chelating agent results in a sharp and prolonged increase in motility and rate of oxygen consumption. Thus in one experiment 5% sperm in sea water respired at a constant rate of 17 $mm.^3$ O_2/hr./ml. sperm, whereas addition of Versene to a concentration of 2×10^{-4} M caused a 5.5-fold increase in rate of oxygen uptake. Even after 90 minutes in Versene the sperm respired at nearly three times the rate of the sea water-treated sperm. Similarly 2×10^{-2} M alanine caused an approximately 3-fold increase in respiration of a 10% sperm suspension.

From these observations it appears likely that the increased motility of starfish sperm treated with chelating agents depends largely upon energy from aerobic metabolism and not upon anaerobic pathways as in sea urchin sperm.

Versene improves fertilization in Asterias to the extent that less Versene-treated sperm are required to achieve a given percentage of fertilized eggs. Thus in one experiment 9 times more

[1] Aided by a grant from the U. S. Public Health Service, and by a grant from Dr. A. Wander, S. A., Berne, Switzerland.

[2] Aided by a grant from the National Science Foundation.

sea water-treated than Versene-treated sperm were required to fertilize 50% of the eggs. This effect may represent a prolongation of the life span of the sperm by Versene.

As expected, metal ions inhibited the sperm mobilizing action of Versene. However, control experiments showed that certain metals in fairly high (0.05 *M*) concentration greatly increased the motility of Asterias sperm. Nickel in the form of $NiCl_2$ was examined most thoroughly. This ion not only increased motility but in 0.02 *M* concentration also increased the rate of respiration 2.5-fold. Cobalt and possibly other metal ions have similar action.

Arterial blood sugar content of toadfish, intact and treated with alloxan or adrenal steroids. PAUL FOLEY NACE.

By latex-injection studies, gill arteries were selected for serial blood sampling and intravascular injection. Fish of 80–700 gms., anaesthetized with chlorobutanol, provided 1 ml. or more of blood, drawn through No. 23 needle, penetrating the artery between and parallel to a pair of gill filaments on the dorsal quarter of the gill. In fish of 200 gms. or more, intravascular injections of 5 ml. were made without leakage.

Anthrone reaction on 0.1 ml. samples determined mean normal value of 73 mg. per 100 ml. of blood, with range 20–185 mg.%, and about 15% of intact fish over 100 mg.%. Typical fish showed 35 mg.% when received, 185 after 4 days heavy feeding on scallops, 65 next day and 95 following day. For intact and treated fish, duplicate samples and samples from heart, hepatic portal tributaries and inter-renal venous sinus showed very similar values.

Alloxan given intravascularly at 500 mg./kg. or subcutaneously at 800 or 1000 mg. was lethal in 3–6 days, with severe terminal edema and terminal blood sugar values of 20–45 mg.%. Following intravascular alloxan at 400 mg./kg., the mean reached 130 mg.% in one day, then fell to intact level. Response to 300 mg./kg. was similar. After these and lower doses, fish survived indefinitely.

After dehydrocortisone (Merck) intramuscularly at 10 mg./day for 6 days, the mean reached 145 mg.%, remained high six days after end of treatment and continued at intact level until sacrificed, six days later. Hydrocortisone (Merck) was lethal to 50% of fish in six days at 10 mg./day, with mean of 113 mg.% after 4 days. After 40 mg. of hydrocortisone in 4 days, one fish reached 210 mg.%, the highest level found after any treatment.

Tissues were fixed for histological examination.

Reversible shrinkage in Chaetomorpha. W. J. V. OSTERHOUT.

Certain compounds consist of long chains of carbon atoms bearing negative charges which repel each other and thus lengthen the chain. When hydrogen ions are added they neutralize these charges and this shortens the chain; consequently there is shrinkage which is reversible. The following experiments on the green cells of the marine alga, *Chaetomorpha Linum* Kützing, indicate that the similar process may occur under certain conditions. On heating the cells in sea water at 55° C. for ten minutes death occurs as shown by the ready penetration of acid fuchsin. When these cells are transferred to sea water at room temperature for one half hour no changes occur. But rapid shrinkage occurs when they are transferred to 0.1 *M* HCl (after a rapid washing in distilled water in which no changes occur). If the shrinkage is not severe it is rapidly reversed when the cells are transferred to 0.1 *M* NaOH (after a rapid washing in distilled water in which no changes occur). The normal dimensions of the cell walls are maintained. This process can be repeated several times on the same cell. Rapid shrinkage occurs below 0.1 *M* HCl until 0.00001 *M* (pH 5) is reached when it occurs more slowly. In distilled water (pH between 5 and 6) the shrinkage occurs after one hour. This is rapidly reversed if not severe in 0.1 *M* NaOH. Similar reversals occur in 0.01 *M* NaOH but the reversal is not so marked in 0.001 *M* NaOH (pH 11).

The following experiments present apparent exceptions to the theory stated above. 1) When cells heated in sea water at 55° C. for ten minutes are placed in a phosphate buffer solution at pH 6 at concentrations between 0.05 *M* and 0.0005 *M* rapid shrinkage occurs; this is somewhat reversible in 0.1 *M* NaOH if the shrinkage is not severe. 2) Some shrinkage occurs if cells are heated in sea water at 55° C. for ninety minutes. This is somewhat reversible in 0.1 *M* NaOH if the shrinkage is not severe. In both cases there is considerable variation.

Irregularities during meiosis in variety 9 of Tetrahymena pyriformis. CHARLES RAY, JR.[1]

Variety 9 of *T. pyriformis* is known only from collections made in Panama and Colombia by Elliott. Two strains representing mating types II and III were studied cytologically. Conjugation was induced readily, but the time between mixing and pairing of mating types (at 24°–25° C.) was twenty-four to thirty-six hours vs. four to five hours for variety 1. Stages of nuclear reorganization during conjugation are like those of variety 1, *i.e.*, three prezygotic and two postzygotic divisions. Meiosis occurs during the first two prezygotic divisions. Counts made during first meiosis suggest five pairs of chromosomes. Definite characterization of the chromosome complement is difficult because many aberrations were seen at MI: chromosome clumping; multivalent chromosome associations; chromatin bridges; and variable numbers of fragments. Nevertheless, formation of pronuclei, reciprocal interchange of migratory pronuclei, synkaryon formation, two postzygotic divisions, and formation of two presumptive macronuclei and two micronuclei, all occur in most conjugants. No breakdown of attachment membranes or massive exchange of cytoplasm between conjugants was seen. When nuclear reorganization is complete, the old, condensed macronucleus is located centrally or anteriorly rather than at the extreme posterior end of the conjugant, which is typical for variety 1. No viable exconjugants have been recovered from numerous isolated pairs.

Studies are in progress to investigate the following questions: are the aberrations typical of all geographic collections of variety 9; is abnormal meiosis a reflection of chromosome structure rearrangement or of some incompatibility between mates; can lethality be related to these abnormalities?

Microscopic observation of reciprocal interchange of pronuclei in Tetrahymena pyriformis. CHARLES RAY, JR.[1]

Living *T. pyriformis* undergoing conjugation were held in a precision microcompression chamber according to Wichterman's technique and observed by means of a phase microscope. The macronucleus, micronucleus, and micronuclear divisions were followed readily. The three prezygotic and two postzygotic nuclear divisions have been observed in their entirety.

The critical placement of the nuclear products after some divisions is a result of orientation and stretching of the anaphase spindles. The placement after other divisions is a result of nuclear migration. The nucleus undergoing the third mitotic division to form the pronuclei is determined by the orientation of the second division spindles, one of the four products coming to lie near or next to the attachment membrane.

The migratory pronuclei of the two conjugants pass each other at different levels of focus in the region of the old mouth parts. There appears to be little or no cytoplasmic exchange during the reciprocal interchange of the pronuclei. No trail or path was seen behind the migratory pronuclei as they passed through the membrane. There appears to be no size or shape difference between the stationary and migratory pronuclei. The pronuclei approach each other, come in contact, and immediately coalesce to form the synkaryon. The second postzygotic division results in two nuclei in the posterior end and two in the anterior end of each conjugant. The anterior nuclei begin enlargement immediately upon completion of anaphase. These become the macronuclear anlagen.

The immaturity period in three varieties of Tetrahymena pyriformis.[2] CHARLES RAY, JR., ALFRED M. ELLIOTT AND GORDON M. CLARK.

Exhaustive studies of variety 1 seem to indicate that sexual immaturity period lasting several weeks exists in *T. pyriformis* following conjugation. During the course of investigating other varieties it became obvious that if an immaturity period occurred it was of very short duration. Therefore a critical study was made of this event in varieties 2, 4 and 6.

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[1] Aided by grants from the National Science Foundation and from The McCandless Fund of Emory University.

[2] This investigation was supported in part by a research grant (PHS G3588) from the National Institutes of Health, Public Health Service.

Fifty pairs from each of the varieties were isolated in peptone broth and at separation one member of each pair was stained to determine whether or not nuclear reorganization had taken place. The remaining exconjugant of each pair which showed macronuclear anlagen was permitted to grow up in a depression slide and then transferred to a tube. Such clones were washed and mated back to the original parental clones and the time of pairing noted.

In variety 2, mating types I and II, pairing occurred when they were tested 5½ days following isolation. In variety 4, mating types I and II, conjugation took place within 3 days after separation of the conjugants. The time of pairing was 4 days in variety 6, mating types I and II. Obviously these tests do not prove whether or not these organisms possess an immaturity period but they do demonstrate that the period is much shorter than that found in variety 1. The length of the immaturity period, or its existence at all, is useful information when working with these varieties.

Influence of roentgen rays on fibrinogen-thrombin interaction. Peter Rieser.

Solutions of purified bovine fibrinogen of varying concentrations in saline buffered at pH 7.4 were irradiated with doses ranging from 500 to 2000 r, and were subsequently clotted at a temperature of 37.5° C. by the addition of purified bovine thrombin. The clotting time (*i.e.*, firm-gel time) of the irradiated fibrinogen was prolonged, the effect increasing with increasing irradiation doses. This occurs at fibrinogen concentrations from 0.95 to 4.75 mg./ml.; at higher concentrations, irradiation produces no clotting delays. The thrombin clotting time of citrated human and bovine plasma is not prolonged until 5000 r are given. Irradiation was found to have no effect on the recalcification time of human and bovine plasma. Thrombin-free human or bovine serum added to irradiated fibrinogen has the effect of preventing the irradiation-induced clotting delay. Preliminary experiments indicate that the rate of solution in 30% urea (final concentration) of fibrin clots obtained from irradiated fibrinogen is accelerated. Apparently, even at the low doses employed, the fibrinogen molecule is altered by roentgen irradiation.

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Chromatophore responses of Uca pugnax to background and total illumination. Muriel I. Sandeen.

Chromatophores of *Uca pugnax* exhibit definite adaptational responses to black and to white backgrounds at light intensities from 3 ft.c. to 240 ft.c. Groups of ten animals each were placed in black containers and in white containers at 3 ft.c., 10 ft.c., 40 ft.c., 120 ft.c. and 240 ft.c. After 1½ hours at each light intensity the average condition of the chromatophores on the legs of the ten animals was determined. Each group of animals was then placed at another light intensity and the average condition determined again at the end of 1½ hours. All experiments were conducted during the day and a total of 180 animals was used. At all light intensities the black chromatophores were more dispersed in animals on a black background than in those on a white background. Similarly the white chromatophores were more dispersed on a white background than on a black background. Responses to increased light intensity in the range studied were found in the black chromatophores of animals on a white background and in the white chromatophores of animals on a black background. The average condition of the black chromatophores on a white background increased from a value of 3.6 at 3 ft.c. to a value of 4.2 at 240 ft.c. The average condition of the white chromatophores on a black background increased from 2.7 at 3 ft.c. to 3.9 at 240 ft.c. These results suggest that there is a significant difference between the responses of the chromatophores of *U. pugnax* and those of *U. pugilator*. It has been shown for the latter species that the response to total illumination dominates the response to background, in that the black chromatophores are more dispersed on a white background than on a black background.

The effect of drying on the performance of the Northrop-Anson diffusion cell. Melvin Spiegel, Evelyn Spiegel and Albert Tyler.

In attempts to get reproducible values of diffusion rates through the scintered glass disc of the Northrop-Anson diffusion cell we have found that drying the cell between successive runs adversely affects the results. A cell of 40 ml. capacity was used in the present work. A series of 10 separate experiments in which 1.0 N HCl was allowed to diffuse into water each gave values for 4 or 5 successive 15-minute intervals that were in agreement to within 2 per cent. However, the average values for each run ranged from 0.168 to 0.318 millimoles of 1.0 N HCl passing through the disc in 15 minutes. The cell had been cleaned and air-dried between each run. When, however, the disc was kept continuously immersed in fluid, then consistent results were obtained. Thus three separate runs with 1.0 N HCl gave values (average of 4 fifteen-minute samples) of 0.266, 0.267 and 0.267 millimoles of HCl diffusing per 15 minutes. The results are interpreted to mean that upon filling the cell air pockets remain in a variable number of channels in the disc and are stable there (Boyle's XIIIth Hydrodynamical Paradox). Thus a different amount of diffusing area would result each time the cell is filled if it has been previously dried.

Influence of the rate and the magnitude of temperature change on the diurnal melanophore rhythm of the fiddler crab, Uca pugnax. G. C. STEPHENS.

Exposure of *Uca pugnax* which have been maintained in darkness at 18° C. to a temperature of 9.5° C. for a period of 12 hours produces a shift in the diurnal melanophore rhythm of two to four hours compared with control animals maintained at 18° C. Experiments were designed to attempt to analyze the characteristics of this response.

Animals were maintained at 31° and at 18° in constant darkness. After an initial period of 18 hours, animals were transferred from 31° to 18°, from 31° to 9.5°, and from 18° to 9.5°. In each case the exposure to the lower temperature was twelve hours. The temporal shift induced by the twelve-hour exposure to 9.5° was the same in the two groups so treated despite the fact that one group was moved to this temperature from 31° and the other from 18°. The transfer from 31° to 18° produced no measurable shift.

Other experiments were concerned with the influence of the rate of change of temperature on this response. Animals were taken from 18° and placed at 9.5° in a large volume of water so that the temperature change occurred gradually over a six-hour period. They were then abruptly returned to 18°. Rapid cooling and slow warming of the animals was also carried out. The response of the rhythm was compared with controls in which both temperature changes were abrupt. Slow cooling of the animals did not diminish the shift obtained and even appeared to increase it. Slow warming of the animals was without any effect on the shift produced.

These results suggest that there is a critical temperature between 18° and 9.5° which must be traversed to induce a shift in the rhythm, and that the rate at which this temperaure is traversed is not critical within the limits of these experiments.

Use of the Mitchison-Swann elastimeter as an aid in micromanipulation. ALBERT TYLER.[1]

Mitchison and Swann (1954) employed a fine pipette attached to an adjustable reservoir to measure elastic properties of sea urchin eggs by sucking the eggs partially into the tube and determining the amount of pressure required to produce deformations of various magnitudes. A simplified version of this can be used as a holder for small eggs and other microscopic objects to be subjected to microdissection or injection. The micropipette is mounted on a micromanipulator and leads to a U-tube, the lower portion of which consists of heavy rubber tubing. The upper portion of the distal side of the U-tube is of glass, for convenience in observing the fluid level, and is mounted on a rack and pinion. The amount of suction, or pressure, produced at the open end of the capillary is readily regulated by lowering or raising the distal arm of the U-tube. The diameter of the open end of the capillary should, in general, be less than two-thirds that of the object to be held, and is dependent on the plasticity and other properties of the object to be held. If one desires rapid change in pressure the capillary should taper rather sharply. For slow pressure change a long slender tip or one with a constriction is used. Fire-polishing usually produces a slightly funnel-shaped opening which offers some advantage over a sharp flat surface in holding the object, and is less likely to damage the object.

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[1] Supported by a grant (C-2302) from the National Cancer Institute, Public Health Service.

By means of this sucker it is possible to operate by horizontal approach under the microscope rather than vertically as in the conventional hanging drop. This permits more precise observation. The frustrations of having the object frequently bounce away upon attempted insertion of the microneedles or micropipettes are also largely eliminated. The object is readily oriented in desired position on the sucker. The sucker permits also operations in dishes containing large quantities of fluid, thus minimizing dangers of evaporation and incidentally damping vibration of the micro-instruments.

Apparent and real micro-injection of echinoderm eggs. ALBERT TYLER[1] AND ALBERTO MONROY.[2]

By use of the sucker described above (Tyler, 1955) for horizontal insertion of micropipettes it is possible to observe closely injection from micropipettes. In experiments on eggs of Echinarachnius and Asterias we have observed that the plasma membrane generally stretches and covers over the pipette (Pyrex or soft glass with tips $< 0.5 \mu$). As the pipette pushes into the egg a funnel-shaped indentation forms, its apex approaching the original center of the egg. Upon further insertion the pipette may appear to have entered the egg cytoplasm. However, generally the plasma membrane forms a long sleeve that is closely adherent to the pipette as the funnel narrows over it. This can readily be shown by injecting colored fluids which expand the sleeve and flow out along it (see also Needham and Needham, 1925). The fluid may temporarily stretch the vitelline membrane, previously pierced by the pipette. It is possible to tunnel completely through the egg (see also Dan, 1943) without the pipette entering the cytoplasm.

To enter the egg cytoplasm proper it is necessary to rupture the remarkably extensible plasma membrane. We accomplished this by allowing the funnel to narrow and the sleeve to adhere tightly to the pipette. One or more light taps on the micromanipulator base cause the tip of the pipette to penetrate into the cytoplasm. Penetration is revealed by the diffusion of dye-containing NaCl-KCl solutions through the cytoplasm. Penetration is also most readily and clearly established by the sudden appearance of a resting potential when a micro-electrode is inserted (see below). A combined pipette and electrode (Grundfest, Kao, and Altamirano, 1954) would increase reliability of injection.

Disappearance of spindles in sand-dollar blastomeres after ultraviolet irradiation of cytoplasm. R. B. URETZ AND R. E. ZIRKLE.

In earlier experiments (with W. Bloom) it was observed that, when an ultraviolet microbeam was directed on cytoplasm of mitotic cells in newt tissue cultures, the spindle disappeared if already present and failed to appear if not yet present; these observations were made by phase contrast microscopy. Similar results have now been obtained with the developing egg of *Echinarachnius parma*. Polarized light was used to observe the spindle, which is strongly birefringent in this species. One blastomere of the two-celled embryo was irradiated; the other served as control, as also did other embryos in the microscope field. When a heterochromatic ultraviolet microbeam was swept around the peripheral cytoplasm of a metaphase blastomere, the birefringent spindle disappeared almost immediately, but not in the controls. Similar bombardment before the spindle was present prevented its appearance, although normally birefringent spindles appeared on schedule in all controls. Asters, as seen by transmitted unpolarized light, were not affected, but, in cells irradiated before spindle formation, transition from monaster to diaster condition was prevented. The control cells cleaved normally, but no cleavage of an irradiated one was observed (among a dozen followed for at least double the normal cleavage time). This is in contrast to the newt, where disappearance or inhibition of the spindle is followed by a "deranged metaphase" of characteristic configuration and often by a "false anaphase," in which two groups of whole chromosomes (not chromatids) move apart, after which cleavage occurs. To observe such results in echinoderm eggs will probably require judicious adjustment of ultraviolet dose and possibly also of wave-length, as well as use of stained preparations in supplement to the living material.

[1] Work of this author supported by a grant (C-2302) from the National Cancer Institute, Public Health Service.

[2] Fulbright Fellow.

On the resistance of Tubularia to x-rays. DONALD J. WATT.

Fifteen–twenty mm. unbranched stems of Tubularia sp. were cut and irradiated in plastic dishes containing 0.25 inch or less of sea water. X-rays were delivered at a rate of 5600 r per minute. Immediately following irradiation the water was replaced by freshly filtered sea water, which was changed daily thereafter, and the stems were kept in covered stender dishes on a sea table at ca. 22° C. The degree of reconstitution was recorded at intervals, and at a designated time complete and incomplete hydranths were removed to permit succeeding reconstitutions. A large proportion of these stems reconstituted twice. The following data are for second reconstitutions: after 2330 r, 10 out of 10 stems reconstituted completely (control, 10 out of 10); after 4660 r, 11/36 reconstituted completely and 5 others partially at the time of cutting (partially = at least striation stage) (control 38/40); 9320 r, 19/40 completely, 3/40 partially (control 36/40); 14,000 r, 15/18 completely, 2/18 partially (control 18/20). In all cases reconstitution was retarded in comparison with controls (about 24 hours behind the latter), but the hydranths had a normal scale of organization for their size, though some were smaller than controls. In no case was reconstitution seen a third time; most of the controls underwent three reconstitutions. Representative stems were examined for interstitial cells, and while their number was diminished, especially with higher doses, there was also a marked thinning of the coenosarc. There was no evidence for a marked differential susceptibility of the I-cells. The fact that, above 2330 r, more stems in the higher dosage groups reconstituted than in the lower invites further investigation.

Survival and other effects following x-irradiation of the flagellate, Euglena gracilis. RALPH WICHTERMAN.[1]

Specimens of the green flagellate, *Euglena gracilis,* which were cultivated in lettuce infusion, were x-irradiated free of air in two types of chambers: two-cc. Nylon syringes and tightly covered two-cc. plastic wells.

Euglena is much more radiosensitive than *Paramecium* and other ciliates. Immediately after irradiation with dosages of 16,500 r and higher, swimming behavior of many of the flagellates becomes markedly altered. Such irradiated specimens, while still active like the controls, swim in a less forward, directional path, occasionally describing circles or swimming short distances only to change direction in an erratic manner demonstrating a kind of "avoiding reaction." Later the irradiated specimens change body shape from the elongate condition characteristic of the normal forms and become shorter and ellipsoidal, frequently exhibiting slow euglenoid movement. Still later, specimens become immobilized and sub-spherical. Before immobilization, specimens may exhibit a vibratile, quivering movement. The immobilized, irradiated flagellates appear more darkly green in color with a more pronounced cell boundary which is a sign of impending death. Dosages greater than 16,500 r hasten these changes.

Upon being exposed to light, successful cultures of *Euglena* can be established after specimens are irradiated with dosages of 55,000 r and less—the smaller the dosage, the more quickly the establishment of the culture. With 55,000 r, the irradiated euglenoids, when placed in culture medium immediately after irradiation, gradually approach almost complete extinction but after the 13th day, the survivors of the irradiation slowly increase in number to produce flourishing cultures. Specimens do not ultimately survive dosages greater than 55,000 r. After irradiation with 64,000 r, survivors remain alive until the 12th day, then die.

The median lethal dose (LD 50) for *Euglena gracilis* appears to lie between 30,000 and 34,000 r at 24 hours.

Carotenoids in the skin of Anolis carolinensis. CHARLES G. WILBER AND ROBERT DARBY.

Despite the fact that many studies have been made on the physiology of color change in reptiles, the carotenoids in this group as a whole have received less chemical attention than in any other vertebrate group. The pigments in the skin of *Anolis* were extracted and separated using solubility, paper chromatography, and column chromatography in the process. Spectral absorption curves were measured and maxima were found at 476 and 506 millimicra. Chemical tests showed that the cartenoid must have an aldehyde, epoxide, or furanoid functional grouping. These data, in conjunction with the solubility properties of the unknown, indicate that the single carotenoid is *rubichrome*. Absorption peaks were also found at 380 to 383 millimicra. Naturally occurring carotenoids are presumed to be of the *trans*-configuration. Apparently, during processing a *cis*-transformation took place and is reflected in the *cis*-peak in the ultraviolet region. The proof of structure of the colored pigment will be unequivocally demonstrated by isolating a sufficient quantity of crystalline material so that elemental analysis and melting point estimations can be made. This latter work is now in progress.

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[1] Part of a study aided by a contract between the Office of Naval Research, Department of the Navy and Temple University (NR 135-263) and the Committee on Research, Temple University.

LALOR FELLOWSHIP REPORTS

The silent period in the optic nerve discharge of Limulus. RODERICK K. CLAYTON.

Action potentials were recorded from small bundles of fibers in the optic nerve of the lateral eye of Limulus; patterns representing one, two, or several active fibers were obtained when areas of the eye of various sizes were illuminated. Steady illumination, initiated abruptly, produced the familiar pattern of an initial high-frequency outburst followed by a steady discharge of lower frequency; under some conditions the initial outburst was followed by a period of inactivity (the silent period). Observations of Hartline and others concerning this silent period were confirmed: the silent period is exhibited only by dark-adapted preparations, and its occurrence is not correlated with a critical frequency or duration of the initial outburst. In a multiple-fiber preparation the silent period occurs synchronously in all fibers, despite varying latent periods and frequencies among the different fibers. Its duration could be diminished slightly by restricting the area of the eye illuminated without altering the intensity of light impinging upon the ommatidium under investigation. The silent period persisted, however, even when the area illuminated was fifty microns in diameter (smaller than a single ommatidium). We conclude that the silent period is a property of the eye as a whole, requiring dark adaptation and enhanced by the simultaneous activity of many receptors (probably as a consequence of mutual inhibition). It is interesting to note that electrical stimulation through the cornea can produce a discharge which displays the silent period. The dependence of this effect upon light adaptation has apparently not been studied.

Metabolism of inorganic nitrogen compounds by marine bacteria. HOWARD GEST.

Part of the nitrogen cycle in sea water involves the sequential oxidation of ammonia to nitrite and nitrate. These oxidations are believed to be catalyzed by marine types of the chemosynthetic autotrophs *Nitrosomonas* and *Nitrobacter*, but a review of the literature indicates that this view requires further experimental support. On the basis of comparative microbiology and biochemistry, it seems possible that certain photosynthetic bacteria might be capable of effecting the reactions in question. Attempts to isolate such organisms from sea water by classical enrichment techniques, however, gave negative results. Considering the low concentrations of nitrite and nitrate encountered in sea water, it appears desirable to investigate the alternative possibility that these compounds are produced by heterotrophic bacteria, particularly since soil organisms other *Nitrosomonas* are known to oxidize ammonia to nitrite. In this connection, a marine *Pseudomonas* containing a high level of nitrate reductase was isolated and is now being examined for ability to oxidize nitrite to nitrate under various conditions.

It is likely that fixation of molecular nitrogen by bacteria is a significant reaction in the marine nitrogen cycle. The common occurrence of photosynthetic bacteria in sea water suggests that these nitrogen-fixers may be of particular importance in this regard. An unusual massive growth of purple photosynthetic bacteria was observed in a large salt water marsh area near West Falmouth and enrichment cultures using the sulfide-saturated purple sand as inoculum showed rapid development of a sulfur purple bacterium (*Chromatium* species). Similar massive accumulations of marine purple bacteria have been observed in European coastal waters, beaches, etc. Studies on the nitrogen metabolism and other aspects of the physiology of the bacterium isolated are contemplated.

Metabolism and function of L-fucose in marine organisms. Maurice Green.

The methylpentose, L-fucose, is a constituent of many brown and green algae and occurs in the jelly coat substance of several species of sea urchin eggs. However, no information is available on its metabolism in these forms. Therefore, the distribution of fucose isomerase was studied in a number of fucose-containing marine invertebrates; this enzyme, found in a mutant of *E. coli,* converts fucose to the ketose, fuculose. The enzyme was not present in unfertilized eggs, fertilized eggs, sperm, ovaries and testes of *Arbacia punctulata* and the brown algae *Fucus vesiculosus, Laminaria agardhii, Ascophyllum nodosum* and *Mesogloia divaricata* with materials prepared under various conditions of tissue extraction. Enzyme assays were carried out in phosphate and borate buffers at pH 8.0 and at pH 5.0 in acetate buffer employing the sensitive cysteine-carbazole procedure to detect the formation of fuculose. In addition, all preparations except *Laminaria* and *Ascophyllum* were tested for the presence of kinases capable of catalyzing the phosphorylation of fucose and fuculose by adenosine triphosphate. Such kinases were absent. As controls on efficiency of tissue extraction, glucokinase activity was demonstrated in Arbacia eggs and testes, adenosine triphosphatase activity was observed in sperm, and protein was readily extracted from *Fucus* and *Mesogloia* (glucokinase activity was absent from these extracts of brown algae). From these results, it appears likely that free fucose or fuculose does not occur *per se.* Instead it is proposed that phosphorylated forms of fucose and fuculose occur as intermediates in the biosynthesis of the naturally occurring fucose-containing mucoproteins and polysaccharides. It is suggested that fuculose phosphate may be first formed by condensation of smaller intermediates. The fuculose phosphate is then isomerized to fucose phosphate which may then polymerize to form polyfucose. Extracts of *Mesogloia* are being examined for such phosphorylated intermediates.

Polysaccharides of marine algae. Ralph A. Lewin.

The free organic matter in sea water has been recognized as an important factor in the ecology of microplankton, but little is known of its source and nature. The following preliminary studies were carried out to determine what polysaccharides are produced by certain phytoplanktonts and littoral algae. The planktonic forms were all isolated and grown in clonal, bacteria-free culture. The littoral algae were collected from the shore. Media were freed from salts, etc., by dialysis, and the polysaccharides were precipitated from concentrated extracts by alcohol. The sugar moieties in acid hydrolysates were tentatively identified by paper chromatography. (**A** = arabinose, **D** = dextrose, **F** = fucose, **G** = galactose, **M** = mannose, **R** = rhamnose, **X** = xylose, **U** = uronic acid, **Z** = unidentified sugar, $\mathbf{R}_f$ exceeding that of rhamnose. Trace amounts indicated by lower-case letters.)

Skeletonema costatum produced no detectable extracellular polysaccharide, but 30% of the dry weight and leucosin, a polyglucose liberated from cells burst in distilled water. *Nitzschia* aff. *ovalis* liberated 5% **GRXu** into the medium; 3% leucosin in cold water; and 6% **FGXr** on extraction in hot water. *Chlamydomonas euryale* nom. prov. liberated 16% **AGmrx** into the medium, and a further 8% on hot water extraction; this polysaccharide contained 20% sulfate. *Platymonas subcordiformis* liberated 6% **Garu** into the medium as soluble polysaccharide, and 5% **GUam** as shed cell-walls (which cannot therefore be regarded as cellulose). *Porphyridium ? cruentum* liberated 50% organic matter into the medium, and a further 25% was extracted by hot water; both polysaccharides contained **DXruz**, but no combined sulphate. The mucilaginous tubes of the littoral diatom *Amphipleura rutilans* dissolved in dilute alkali, though not in hot water; they consisted of **MXru** with 15% sulphate. The water-soluble mucilages of certain slimy brown algae proved all to be fucoidins with 15–30% sulphate; the species examined were *Mesogloia divaricata* (**Fz**), *Chordaria flagelliformis* (**Fg**), *Leathesia difformis* (**Fgx**), and *Chorda filum* (**Fgxu**).

In vitro ACTH stimulation of incubated frog adrenals. I. A. Macchi.

Adrenal cortical hormone biosynthesis *in vitro* by frog adrenal tissue was investigated using as indices of biosynthesis the blue tetrazolium (B.T.) reducing properties of the α Ketol steroids and response to adrenocorticotropin (ACTH).

Approximately 100 mgms. minced adrenal tissue pooled from contralateral glands of 4–5 American bullfrogs (*Rana catesbiana*) were incubated in 2 ml. amphibian Ringer-phosphate-glucose (200 mgm.%) medium (pH 7.4) in 100% oxygen at 25° C. for one hour without ACTH and for two additional hours with Armour bovine ACTH (10 I.U./100 mgm.) added to one flask of each set containing contralateral glandular tissue. As a control renal tissue obtained from adrenal donors was similarly treated. Reducing lipids were extracted from tissue incubating medium with purified methylene chloride which permits recovery of 98 ± 4.18% hydrocortisone in concentrations of 2.5 and 5 μg./ml. from 2 ml. of Ringer medium and were measured in dried extract residues by the blue tetrazolium procedure of Mader and Buck. Lipid extract residues were not purified consistently since silica gel chromatographic fractionation procedures for corticosteroids did not alter significantly the B.T. values.

Adrenal reducing lipid values expressed as μg. hydrocortisone equivalent/hour incubation/100 mgm. tissue (wet weight) obtained in six experiments averaged 1.23 ± 0.11 in the absence of ACTH and 2.37 ± 0.46 in the presence of ACTH, representing approximately a twofold increase due to ACTH stimulation. In six similar experiments with renal tissue the average lipid reducing values obtained were 0.50 ± 0.21 μg./hr./100 mgm. in the absence of ACTH and declined consistently in the presence of ACTH averaging 0.30 ± 0.23 μg./hr./100 mgm. Adrenal stimulation by ACTH in these studies was obtained consistently with a medium calcium concentration of 2.0 mM./liter but was not observed when the medium calcium concentration was reduced to 1.09 mM./liter. In four experiments in which the latter calcium concentration was employed reducing lipid values averaged 1.38 ± 0.12 μg./hr./100 mgm. in the absence of ACTH and 1.37 ± 0.11 μg./hr./100 mgm. in the presence of ACTH.

Studies on the functions of intracellular ribonucleases. JAY S. ROTH.

If the mechanism of synthesis of protein enzymes involves the participation of RNA, it is probable that at some stage in this synthesis nucleoprotein complexes are formed. There is considerable evidence for the existence of such complexes, the enzyme-protein portion of which is inactive while bound to the RNA. Release of enzyme may be brought about by the action of intracellular ribonucleases (RNases) which degrade the RNA of the ribonucleoprotein. As evidence, the release of protease activity from *Arbacia* egg homogenates by RNase has been reported, and in *Tetrahymena pyriformis* homogenates and rat liver mitochondria, increases in enzyme activity have been observed on addition of crystalline pancreatic RNase. In order to evaluate this theory further, the effect of crystalline RNase on several enzyme activities in unfertilized *Arbacia punctulata* egg homogenates was determined. *Arbacia* eggs were chosen since they represent a system likely to have an accumulation of ribonucleoprotein complexes. However, no increase in the activity of fumarase, deoxyribonuclease, L-leucineamidase or L-benzoylarginine-amidase was observed in egg homogenates after incubation with crystalline pancreatic RNase. Assays were also carried out for RNase activity in egg homogenates and fractions prepared from them, and in sperm. RNase was determined by a spectrophotometric method. One unit of activity is defined as a change in O.D. of 0.001 under standard conditions. RNase activity in eggs measured at pH 7.8 and 5.8 averaged 17.1 and 68.2 units/mg. N, respectively, while in sperm at pH 7.8 it was 125.2 units/mg. N. No evidence was found for a RNase inhibitor in the supernatant or particulate fraction prepared from homogenized eggs centrifuged at 60,000 *g* for 1.5 hours. The evidence, therefore, does not support the above theory, although it is entirely possible that certain key enzymes, not tested for, could be released from nucleic acid combination by RNase.

LIGHT AND ELECTRON MICROSCOPE STUDIES ON THE LIGHT ORGAN OF THE FIREFLY (PHOTINUS PYRALIS)[1]

H. W. BEAMS AND EVERETT ANDERSON

Department of Zoology, State University of Iowa, Iowa City, Iowa

Numerous studies on the light organ of the firefly have been made in an effort to better understand the mechanism of light production. Among these studies the works of Lund (1911), Dahlgren (1917), Hess (1922) and especially Buck (1948) are of interest because, in addition to a detailed description of their own research, they give extensive references to other works in this field. An excellent correlation of the anatomical, physiological and biochemical studies on bioluminescence has recently been the subject of a book by Harvey (1952).

In this paper little can be added to the rather complete and accurate histological analysis available on the light organ of fireflies (Buck, 1948). However, in this organ certain structures beyond the resolving power of the light microscope have been revealed, particularly within the tracheal end cell, which may prove to be important in yielding a better understanding of the mechanism of light production in the firefly. The present report deals with the study of these.

MATERIAL AND METHODS

The material for this study consists of the light organ of locally collected male fireflies, *Photinus pyralis*.[2] The organisms were killed, the light organs immediately dissected and cut into small pieces. Some of the pieces were fixed in Bouin's solution and subsequently stained in Heidenhain's hematoxylin or Mallory's triple stain; some were fixed in Champy's solution, bleached and also stained in Heidenhain's hematoxylin; some were fixed and stained by the method of Regaud for the demonstration of mitochondria; some were fixed in cold acetone and subsequently treated according to the method of Gomori (1941) for the demonstration of alkaline phosphatase; and, finally, some of the pieces were fixed in one per cent buffered osmic tetroxide solution (pH 7.25) after the method of Palade (1952) for study with the electron microscope. These last were then washed, dehydrated, infiltrated and embedded in a mixture of 72% N-butyl methacrylate and 28% methyl methacrylate. Added to this methacrylate mixture was a catalyst, consisting of 0.2 gm. of "Luperco" per 10 cc. of the mixture. Polymerization was accomplished in an oven at 45° C. for 12 to 24 hours. Sectioning was done with an Interna-

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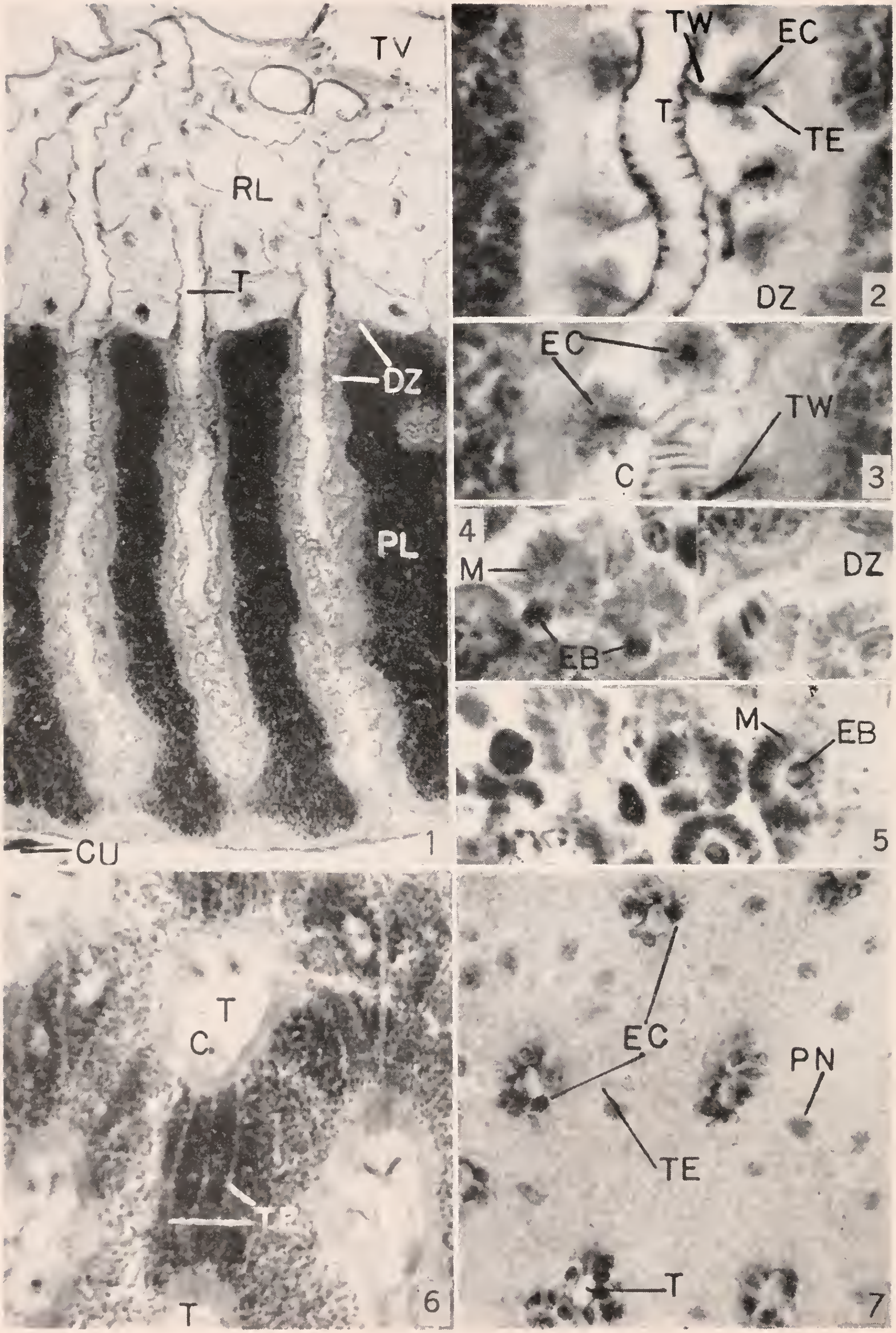
[1] Aided by Public Health Service Grant N. B-301 (C). Thanks are due Mr. Newtol Press for technical assistance and Dr. T. C. Evans for kindly allowing us the use of the electron microscope which was purchased by a grant from the Iowa Division of the American Cancer Society.

[2] We are indebted to Mr. T. J. Spilman of the Section of Insect Identification, U. S. Department of Agriculture, Washington, D. C., for kindly determining for us the species of firefly used in this study. This organism was erroneously referred to as *Photinus scintillans* in a forthcoming abstract of this paper (*Anat. Rec.*, **122**: 483, 1955).


TV
RL
T
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DZ
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1
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3
4
M
EB
DZ
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5
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TB
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6
EC
EC
PN
TE
T
7

FIGS. 1–7.

tional Minot rotary microtome fitted with a glass knife and set to cut at 0.025 of a micron. Observations were made with an RCA model EMU electron microscope.

DESCRIPTION

Light microscope observations

The histology of the light organ in the firefly is well known. However, it seemed desirable to include a plate of photographs illustrating its microscopic anatomy here in order to facilitate the interpretation and correlation of its structure as previously described with that seen in the electron micrographs. Histologically the light organ is composed of two cellular layers, a dorsal layer of refractile cells containing urate granules (Fig. 1, RL), and a ventral layer of deeply-staining, light-producing cells, the photogenic layer (Fig. 1, PL). Outwardly the photogenic layer is covered by a thin hypodermis which is overlaid by a chitinous cuticle. Only a small portion of these layers is shown in Figure 1, CU.

The main ventral transverse tracheal trunks (Fig. 1, TV) are greatly enlarged in the region of the light organ where they give off numerous vertically arranged tracheal branches (Fig. 1, T) that extend down through the layers of the photogenic organ (see also Hess, 1921). This rich supply of tracheae within the photogenic organ is presumably necessary to carry the oxygen thought to be required for the production of light (Harvey, 1953). As the tracheae penetrate the photogenic layer, they extend vertically into tunnel-like regions between the photogenic cells (Figs. 1 and 2, T). These regions (termed "cylinders" by Buck, 1948) are bounded by the differentiated zones of the photogenic cells (Figs. 1 and 2, DZ). The layer of differentiated photogenic cell cytoplasm is relatively refractile to hematoxylin staining when fixed in Bouin's solution (Figs. 2 and 3, DZ). However, by Regaud's method small but well defined bodies which stain like mitochondria are observed concentrated within it (Fig. 4, DZ). If mitochondria exist

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FIGURE 1. Vertical section through part of the light organ which is composed of two layers, the reflector, or urate (RL), and the photogenic (PL). The tracheae (T) are seen to branch from the large transverse trachea (TV) and extend down through the urate and photogenic layers. A small portion of cuticle overlying the photogenic layer is seen at CU. × 400.

FIGURES 2 AND 3. High power view of vertical trachea (T) showing cylinder (C), tracheal twig (TW), tracheal end cell (EC) and tracheoles (TE). The differentiated zone of the photogenic cells is seen at DZ. Dark spindle-shaped photogenic granules are also present in these figures. × 800.

FIGURE 4. Regaud's-stained longitudinal section through three tracheal end cells. Note tracheal twig in center of end cells, radiating villi containing darkly stained mitochondria (M) and the densely stained end bulb (EB). At the upper right of Figure are seen small granules (mitochondria) within the differentiated layer of the photogenic cells (DZ). A transverse section of an end cell is seen at the extreme lower right in figure. × 1000.

FIGURE 5. Tracheal end cells stained as in Figure 4, but cut transversely. Note darkly stained intima of tracheal twig in center of cells and numerous radiating darkly staining villi containing mitochondria. × 1000.

FIGURE 6. Horizontal section through photogenic layer. Tracheoles (TE) are seen extending between two trachea (T) and associated cylinders (C). × 600.

FIGURE 7. Horizontal section through photogenic layer treated by Gomori's technique for the demonstration of alkaline phosphatase. Note reaction in tracheal end cells (EC), photogenic cell nuclei (PN) and a faint reaction of the tracheoles (TE). × 400.

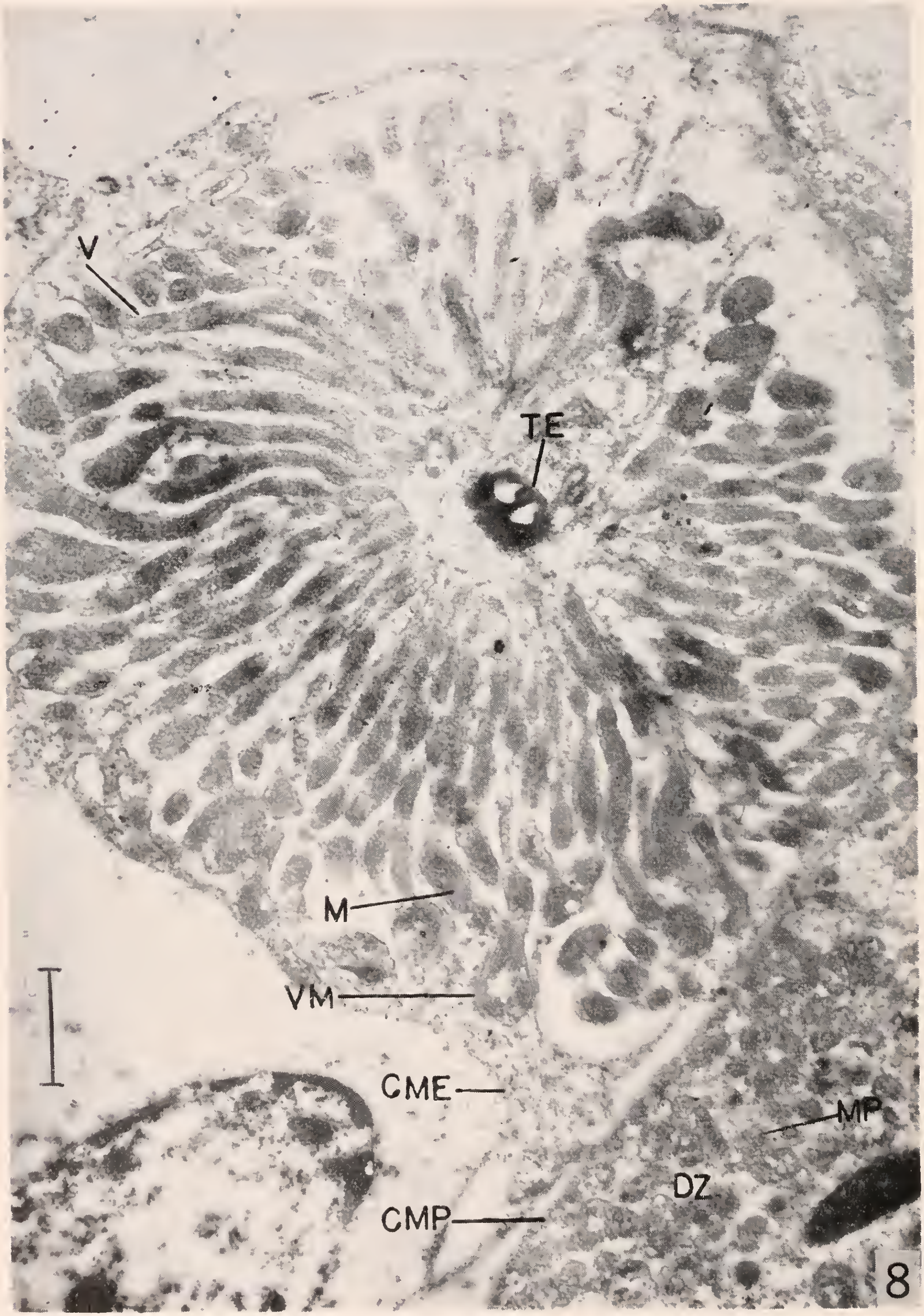
V
TE
M
VM
CME
CMP
MP
DZ
8

Fig. 8.

more deeply within these cells, namely among the photogenic granules, as claimed by Takagi (1934), they could not be demonstrated by this method.

The tracheal twigs (TW) radiate for a short distance within the cylinder where they eventually penetrate, or, at least, become enclosed within the tracheal end cell (Figs. 2, 3 and 4). In Regaud's preparations, radiating from the region of the tracheal twig within the end cell may be seen many filamentous bodies (Figs. 4 and 5, M). Because of their staining reaction and their internal structure displayed in electron micrographs later to be described, these bodies are considered to be mitochondria. As will also be described later, each of the filamentous mitochondria is enclosed in a villus-like membrane structure of the end cell which is not resolved in light microscope preparations.

Near the point where the tracheal twig emerges from the end cell and divides, giving rise to tracheoles, is observed, in tissue treated by Regaud's method, a small rounded body (Figs. 4 and 5, EB). This body is densely stained so that details of structure within it cannot be determined. It probably is the same body as the one described by Dahlgren (1917) in the light organ of *Photinus consanguineus,* although its appearance is somewhat different—which may possibly be due to a difference in the species of firefly investigated, to the degree of destaining of the preparation, or to a difference in general staining procedures. We have termed it the end bulb.

We have not observed the "rounded body" described by Dahlgren (1917) and illustrated by Buck (1948) as S in Figure 11.

Buck (1948) thought the cylinder in life probably contains a gelatinous substance. Nuclei are often seen within it. These probably represent the nuclei of tracheal epithelial cells (Fig. 1).

Where the tracheal twigs emerge from the tracheal end cells they usually branch into two tracheoles which extend between the photogenic cells (Fig. 2, TE). In fact, they sometimes appear to connect neighboring cylinders (Fig. 6, TE).

Preparations of the photogenic layer made by means of Gomori's technique for alkaline phosphatase show a darkening of the nuclei and end cell cytoplasm within the cylinder (Fig. 7, EC). Faint darkening of certain of the tracheoles extending between adjacent tracheal cylinders can sometimes be observed (Fig. 7, TE). The nuclei of the photogenic cells are also darkened by this method (Fig. 7, PN).

Electron microscope observations

1. Urate layer

A typical electron micrograph of the elements of the reflector (urate) layer is shown in Figure 18. The so-called urate granules (UG) appear to possess a finely granular structure. Small vacuole-like bodies are often present near the periphery of the granules. Whether or not these are artifacts due to a contraction of the granular content upon fixation, or to material dissolved out of the granule, is difficult to determine. However, we are inclined to accept the former view. Scattered among the urate granules in the finely granular cytoplasm are numerous fila-

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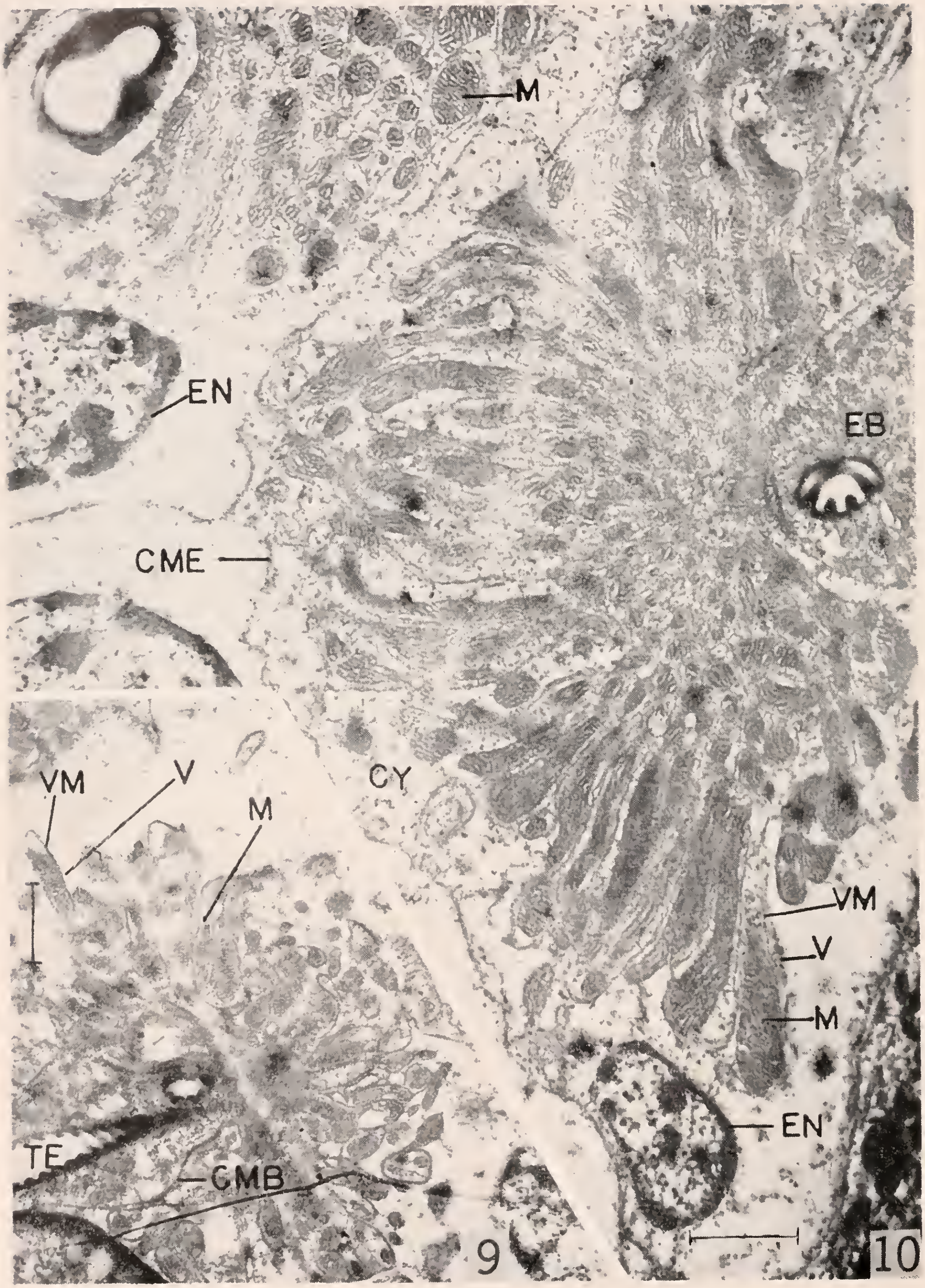
FIGURE 8. Nearly cross section of tracheal end cell. The centrally located tracheoles are seen at TE, protoplasmic processes at V, mitochondria inside of villi at M and membrane covering villi at VM. Surrounding the end cell is a membrane (CME). Mitochondria (MP) are seen located in the differentiated cytoplasmic layer (DZ) of adjoining photogenic cells.


M
EN
EB
CME
CY
VM
V
M
TE
CMB
9
VM
V
M
EN
10

Figs. 9–10.

mentous mitochondria (M). These display striking transverse lamellae which appear to be in reality disc-like structures.

2. Photogenic layer

a. Tracheal end cell

It is difficult to piece together even from many electron micrographs clear evidence as to the exact form and structure of the tracheal end cell. However, evidence from light microscope photographs and electron micrographs indicates that the tracheal end cell is a relatively spherical, or oval-shaped body through the center of which passes the tracheal twig. The end cell possesses a definite outer limiting membrane (Figs. 8 and 10, CME) and a well defined nucleus (Figs. 9, 10 and 13). The tracheal end cell membrane adjoining the centrally located tracheal twig (Figs. 8, 9 and 14) is probably arranged somewhat like the cell membrane at the base of certain kidney cells (Sjöstrand and Rhodin, 1953), Malpighian tubule cells of certain insects (Beams, Tahmisian and Devine, 1955) and certain cells in the green gland of the crayfish (Beams, Anderson and Press, 1955). That is, the tracheal end cell membrane adjoining the tracheal twig is thrown into a multitude of folds resembling villi that radiate into the cell for a considerable distance where they approach, but apparently never become attached to, the outer tracheal end cell membrane (Figs. 8 and 10, CME).

Figure 8 represents an electron micrograph of a nearly transverse section through the distal end of a tracheal end cell. The doubleness of the tracheoles plus the dense material about the intima of them indicates that they are in the process of, or have just completed, emerging from the end cell. This cell displays numerous protoplasmic processes, or villi (V), radiating from the region of the intracellular tracheole (TE).[3] That they have the form of villi is demonstrated in the cross sections where they appear circular or elliptical (especially M at top of Fig. 10). The villi, which seem to be slightly smaller in diameter at the region adjoining the tracheole than they are at their distal ends (Figs. 8 and 10, V), are laden with bodies that appear to be mitochondria (M). The membranes of the villi are shown in Figures 8, 10, and 11, VM.

The conclusion that the bodies filling the villi are mitochondria rather than muscle-like fibers is based upon a study of their internal structure, which closely resembles the morphology of mitochondria as hitherto reported (Palade, 1953; Sjöstrand, 1953; Beams and Tahmisian, 1954). However, variation in the internal structure of mitochondria within different end cells does occur, with the mito-

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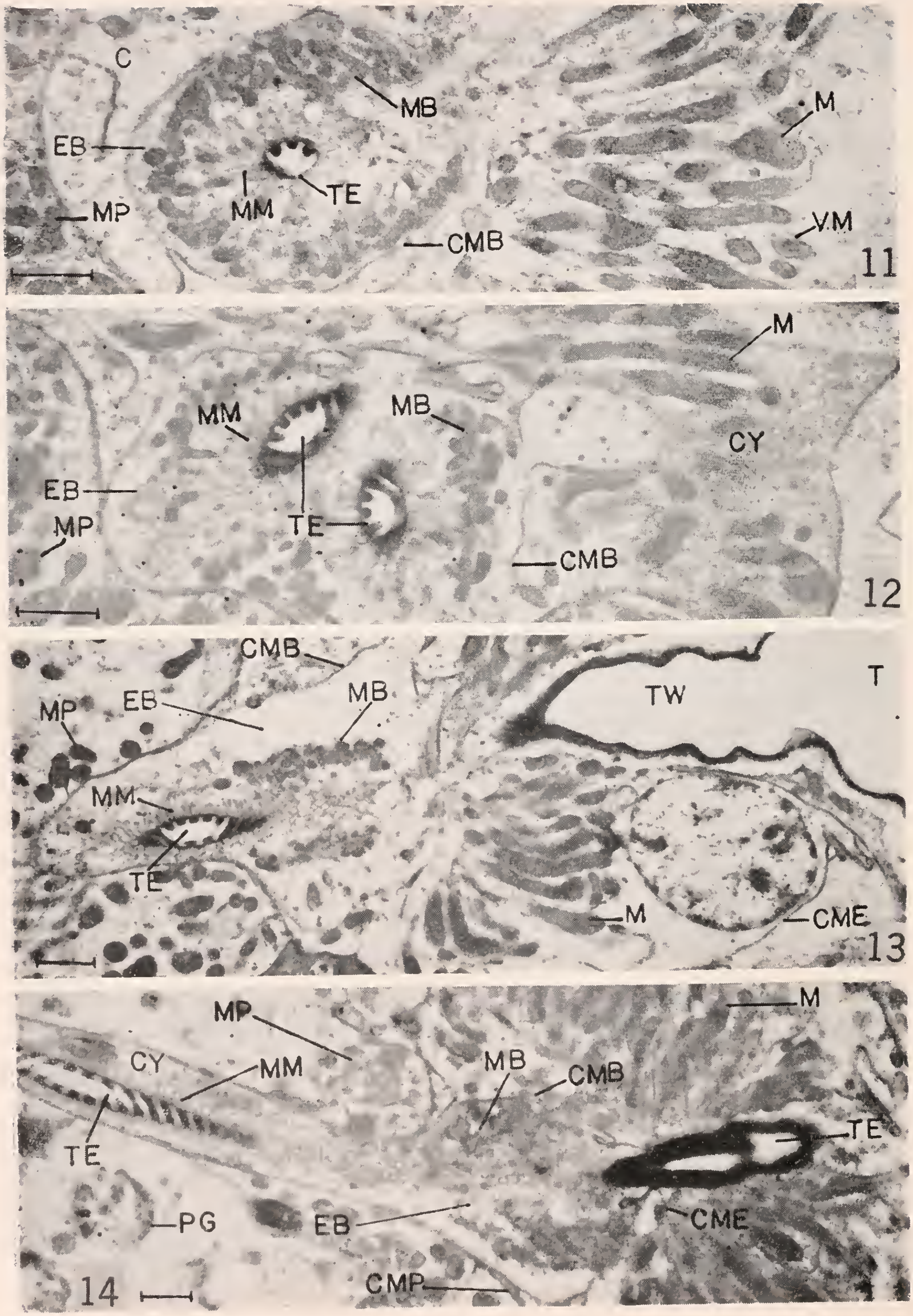
[3] Because of their shape the term villus has been used loosely here to describe the multitude of mitochondrial containing finger-like structures of the tracheal end cells. However, these bodies may differ from villi in being formed by a tubular invagination rather than an evagination of the cell membrane.

FIGURE 9. Nearly longitudinal section through tracheal end cell. Villi (V) with limiting membrane (VM) and mitochondria (M) displaying transverse lamellae. A portion of the end bulb cytoplasm and membrane (CMB) is seen surrounding the tracheole (TE) as it leaves the region of the cylinder.

FIGURE 10. Tangential section through end cell. Note mitochondria (M) with longitudinally arranged lamellae inside villi (V). Each villus is surrounded by a membrane (VM). In the end cell cytoplasm (CY) is the nucleus (EN). The cell membrane is seen at CME. Transverse sections of mitochondria show the lamellae to extend completely across them (M at top of figure).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MP
EB
MB
MM
TE
M
CME
13
M
MP
CY
MM
MB
CMB
TE
TE
PG
EB
CME
14
CMP

Figs. 11–14.

chondria of some of the tracheal end cells bearing longitudinally arranged lamellae (Figs. 8 and 10, M), while those of certain other end cells may display transverse lamellae (Figs. 9 and 16, M).

In Figure 10 is seen a tangential section through a portion of a tracheal end cell. Actually in this section the tracheal tube has emerged from the distal part of the end cell. Because of the rounded shape of the cell and the radial arrangement of the internal villi, the distal portions of the latter appear in this preparation to be cut longitudinally and the proximal portions obliquely. Illustrated here, in addition to the tracheal end cell membrane (CME) and end cell nucleus (EN), are many intracellular mitochondrion-laden (M) protoplasmic processes, or villi (V). The membranes at the surface of the villi (VM) and the end cell cytoplasm (CY) are also shown. The longitudinal sections through the outer extremities of the protoplasmic villi show striking longitudinally oriented lamellae within the mitochondria (M). It will be observed that the extremities of the villi are free and unattached within the end cell, a condition which favors the view that the bodies in question are not contractile elements. Surrounding the tracheole (TE) is a portion of the end bulb (EB) later to be described.

Other sections that are more nearly longitudinal through the end cell are illustrated in Figures 9, 13, 14 and 16. In Figure 9 the radiating villi (V) show mitochondria (M) that possess transverse lamellae similar to those that have been described as cristae by Palade (1953) or as double membranes by Sjöstrand (1953). Figure 16, in addition to showing the usual structures of the tracheal end cells (intracellular villi, nucleus, mitochondria, differentiated layer of photogenic cells), displays the arrangement and distribution of the tracheoles.

In Figures 13 and 14 it is clear that the radiating mitochondrion-filled villi are confined to the tracheal end cell and do not constitute a part of the end bulb.

b. End bulb or "rounded body" of Dahlgren

Dahlgren (1917) described in *Photinus consanguineus* "a rounded and still denser mass of a similar material that embraces the tracheoles just after they leave the end-organ." As previously described, a similar body has been observed by us in Regaud's preparations (Fig. 4). It is more difficult to destain than any of the parts of the end cell. Like the components (villi) of the end cell, Dahlgren (1917) thought this body to be composed of muscle-like fibers that might convey to it powers of a valve-like action. It is impossible, because of its small size, to determine the relationship, if any, between the end cell and the body we here propose to call, for want of a better term, the end bulb (Figs. 11 to 14, EB). Consid-

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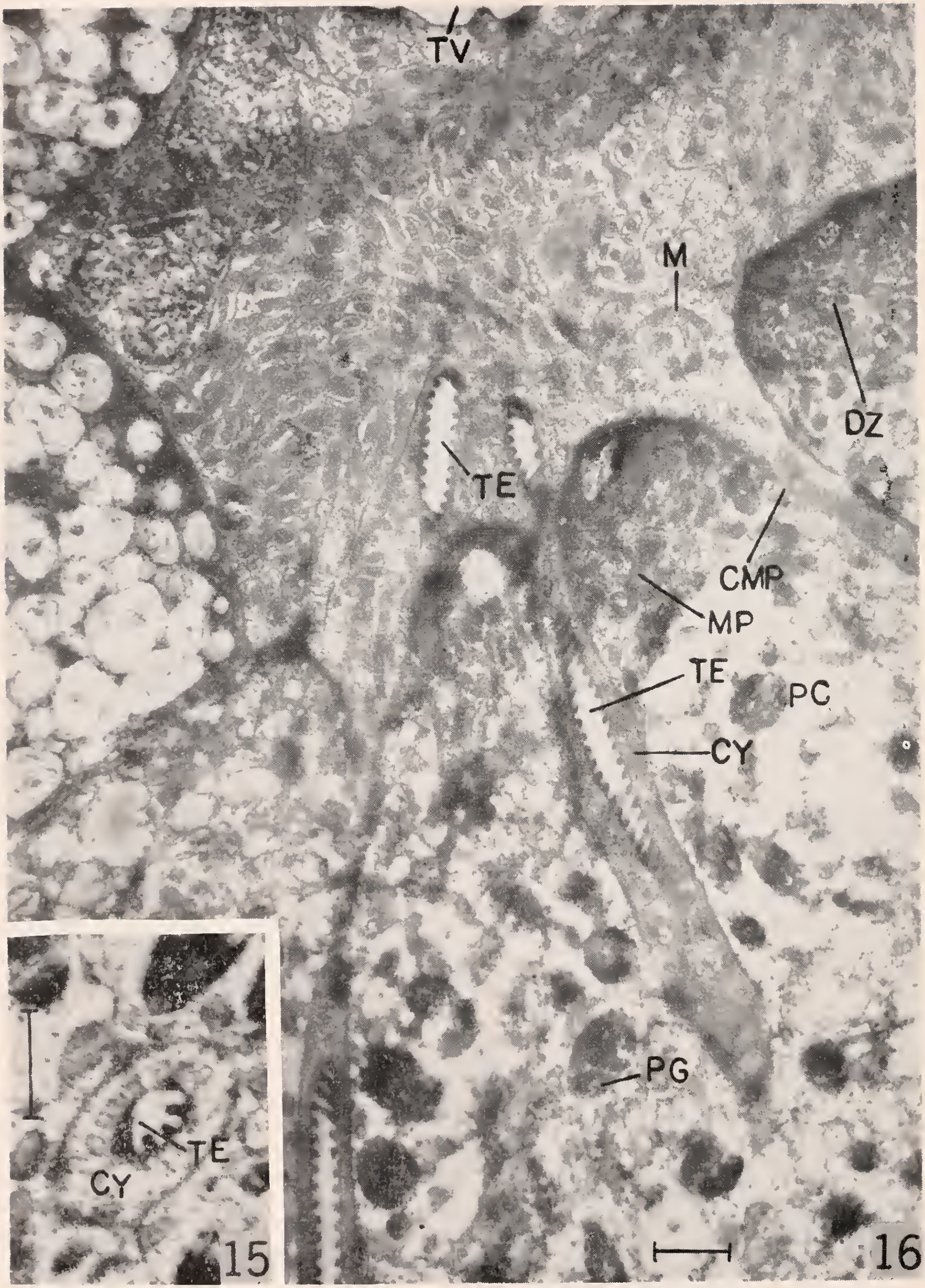
FIGURE 11. Transverse section through end bulb (EB) and part of end cell showing tracheole (TE), microvilli (MM), mitochondria (MB), end cell membrane (CMB), cylinder (C), mitochondria of differentiated outer layer of photogenic cells (MP), end cell mitochondria within villi (M) and villus membrane (VM).

FIGURE 12. Same structures present as in Figure 11, except that here the section was made near the distal end of end bulb. The tracheal twig has divided into two tracheoles (TE).

FIGURE 13. Nearly longitudinal section through end bulb and part of end cell showing trachea (T), tracheal twig (TW), end cell nucleus, villi of end cell containing mitochondria (M), end cell membrane (CME), end bulb membrane (CMB), mitochondria of end bulb (MB), microvilli (MM), tracheole (TE) and mitochondria of photogenic layer (MP).

FIGURE 14. Same structures present as in Figure 13, except that there are two tracheoles (TE) and a well defined end cell membrane (CMB). The form of the villi in the end cell is well illustrated here. The dense bodies within the villi are mitochondria.


TV
M
DZ
TE
CMP
MP
TE
PC
CY
PG
TE
CY
15
16

Figs. 15–16.

erable difficulty was involved in obtaining suitable sections to reveal the relationship between the tracheal twig, tracheole, end cell and end cell bulb. However, Figures 13 and 14 show nearly longitudinal sections and Figures 11 and 12 nearly transverse sections through them, illustrating the initial branching of the tracheoles (TE). Parts of end cells with their radiating mitochondrion-laden villi (VM) and the tracheal end cell bulbs (EB) are illustrated in these figures. At this point it is of interest to compare the electron micrographs in Figures 11 and 12 with the light microscope picture in Figure 5. Compare also Figures 13 and 14 with Figure 4. The question of whether or not the end cell bulb should be considered a part of the end cell, or as an independent cellular structure, is difficult to answer. It will be noted that what appears to be a part of the distal end cell membrane seems to contact the darkened part of the tracheole (intima) at Figure 14, CME. Also near this point is seen the origin of the membrane that surrounds the end bulb (Fig. 14, CMB). However, it cannot be determined for certain, in any of our preparations, whether or not the end cell membrane merely dips in to touch the tracheole at this point in a manner comparable to the behavior of the neurilemma at the node of Ranvier, or whether the two membranes are discontinuous and should be thought of as independent structures. It may be significant that, although considerable cytoplasm is present in the end bulb, we have never observed the presence of a nucleus within it. In any case, the limiting membrane of the end bulb is observed (Figs. 9, 14, and 19) to be continuous as the limiting membrane of the tracheoles.

The tracheal twig penetrates the end bulb at its center much in the same way as it does the tracheal end cell. At the proximal end of the end bulb, *i.e.*, where the tracheole first enters, it appears single (Figs. 11 and 13, TE); more distally, the tracheal elements appear double—which indicates that branching of the tracheal elements occurs within or just after they have emerged from it (Figs. 12 and 14, TE). Extending from the region of the intima of the centrally located tracheole are radially oriented cytoplasmic structures (microvilli) approximately 2000 Å in length (Figs. 11–14, 19, MM). They do not seem to be limited to the end bulb part of the tracheole, but instead may be observed along the tracheolar surface even after it has penetrated well between the photogenic cells (Figs. 14 and 19, MM). When cut in cross section these structures display a reticular or net-like structure (Figs. 13, 14 and 19).

Between the outer border of the microvilli and the membrane of the end bulb (CMB) are located numerous short rod-like mitochondria (Figs. 9, 11–14, 19, MB). These are interpreted as mitochondria on the basis of their form and internal laminated structure. They are not evident in light microscope preparations because of their relatively small size and the difficulty of properly differentiating

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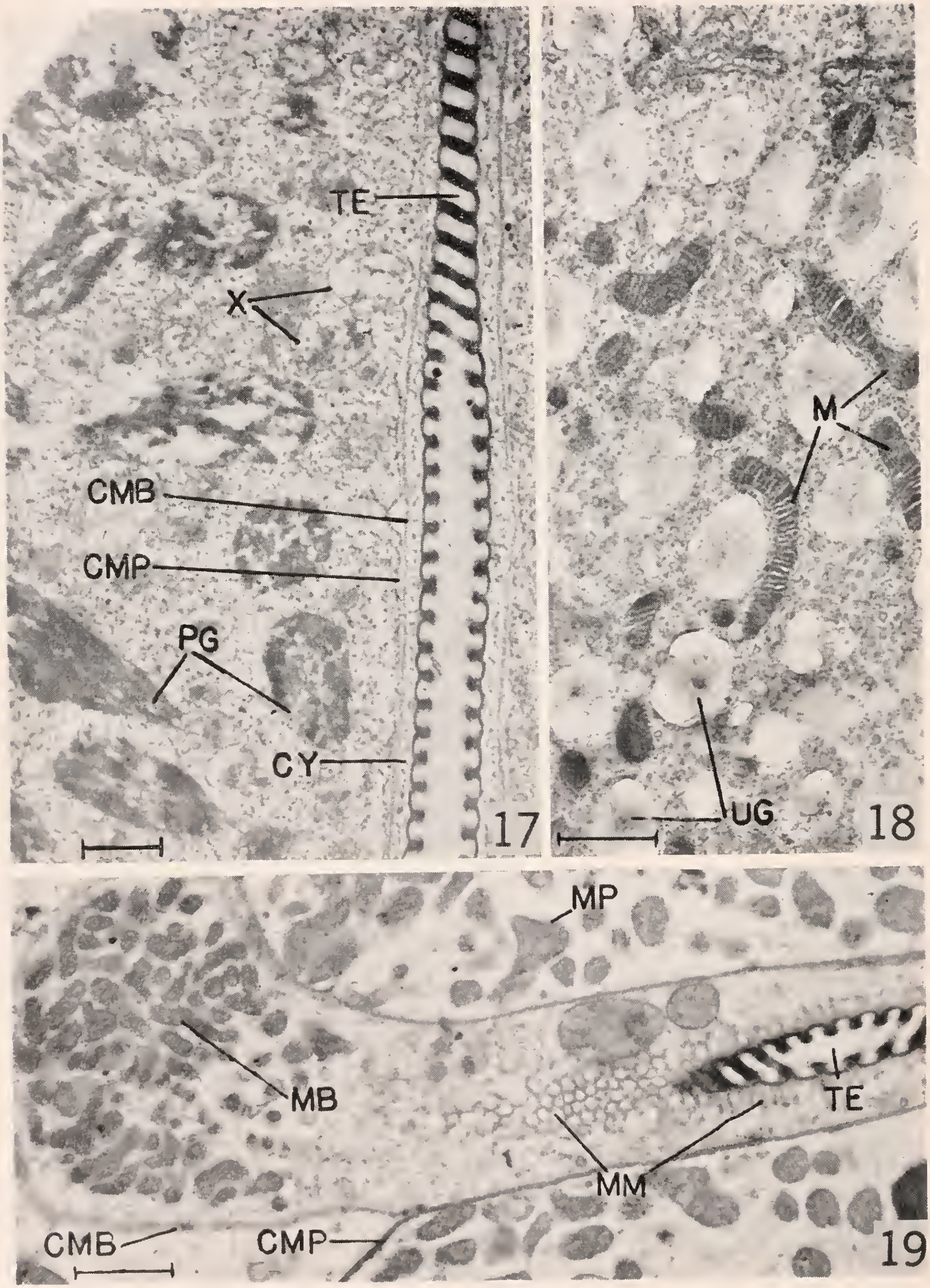
FIGURE 15. Transverse section of tracheole (TE) located between photogenic cells. The tracheole is surrounded by cytoplasm (CY), from the end bulb. The membrane is made up of both the end bulb membrane and photogenic cell membrane.

FIGURE 16. Slightly tangential section through end cell, but showing nearly longitudinal section through tracheoles (TE). A small portion of a large transverse trachea is seen at TV. Villi and mitochondria are cut at various angles. Tracheoles (TE) extend between adjacent photogenic cells (PC). Surrounding each tracheole is end bulb cytoplasm (CY), end bulb membrane and membrane of photogenic cell (CMP). The outer differentiated cytoplasmic zone of the photogenic cells (DZ) contains many granular mitochondria (MP). Lamellar-like photogenic granules are seen at PG.


TE
X
CMB
CMP
PG
CY
17
M
UG
18
MP
MB
TE
MM
CMB
CMP
19

Figs. 17–19.

them when stained. It will be noted that the mitochondria are limited to the region of the end bulb and only now and then a few isolated ones may be seen in the cytoplasmic regions of the tracheoles.

c. Differentiated outer cytoplasmic zone of the photogenic cells

As described in the light microscope preparations, there exists a differentiated outer cytoplasmic zone on the free surfaces of the photogenic cells. This zone seems to be present only on those surfaces of the cells that are situated along the dorsal and ventral surfaces of the photogenic layer and those surfaces of the photogenic cells that border on the tracheal cylinders. Portions of this zone are illustrated in Figures 8, 16 and 19. It possesses a continuous relatively dense outer membrane (CMP). Below this membrane is an area containing numerous granules and short rod-shaped mitochondria (MP). This zone is remarkably free of photogenic granules in spite of the fact that there is no membrane separating the region containing the photogenic granules from that of the mitochondrial-differentiated zone.

d. Tracheoles

As has been described many times, the tracheoles are derived from a branching of the tracheal twig as it emerges from the tracheal end bulb. The membrane covering the end bulb seems to be continuous with that of the tracheoles as they penetrate the region between the photogenic cells (Figs. 14 and 19).

The cytoplasm, too, seems to be continuous with that of the tracheoles (Figs. 14 and 19). However, mitochondria have not been consistently demonstrated within the surrounding cytoplasm of the tracheole beyond the region of the end cell bulb (Figs. 14, 16 and 19). The two bodies seen in the tracheal cytoplasm are unidentified; they may be mitochondria (Fig. 19). The end bulb microvilli are seen in parts of the tracheoles that are entering between the photogenic cells (Figs. 14 and 19, MM).

All the tracheal elements, even the very smallest, possess taenidia (see also Richards and Korda, 1950). We have never observed tracheoles to penetrate the cells of the photogenic layer. Tracheoles located between photogenic cells are covered by at least two membranes—namely, the surface membrane of the tracheole (CMB) and the surface membrane of the photogenic cells (CMP). The relationship of these two membranes is clearly seen in Figures 14 and 19 where they meet and subsequently give the appearance of a single membrane at the surface of the tracheole (Fig. 15). However, they may be differentiated even in tracheoles located deep between the photogenic cells (Fig. 17, CMB and CMP). Furthermore, in this figure the cytoplasmic layer is reduced in size and is free of mitochondria. The differentiated zone of the photogenic cells is also absent in this region. We

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FIGURE 17. Section of tracheole (TE) lying between photogenic cells. Surrounding the tracheal twig are end bulb cytoplasm (CY), end bulb membrane (CMB) and photogenic cell membrane (CMP). Spindle-shaped laminated photogenic granules are seen cut in longitudinal and cross section (PG). Unidentified bodies are observed at X.

FIGURE 18. Section through reflector layer. Note refractile (urate) granules (UG), mitochondria with transverse membranes (M) and finely granular cytoplasm.

FIGURE 19. Longitudinal section through portion of end bulb and tracheole. The end bulb contains numerous short filamentous mitochondria (MB). Surrounding the tracheolar intima and radiating from it is a multitude of microvilli (MM). Where cut in transverse section they give a net-like appearance.

have not observed branching or anastomosis among the tracheoles of the photogenic layer.

e. Cytoplasm of photogenic cells

The most conspicuous structures of the cytoplasm of the photogenic cells are the photogenic granules. These bodies are relatively dense, somewhat spindle-shaped and possess longitudinally oriented lamellae (Figs. 16 and 17, PG). Other bodies, like those at X in Figure 17, appear among the photogenic granules. These bodies have a structure different from that of mitochondria. Possibly they represent a stage in the formation or disintegration of the photogenic granules. As previously indicated we have not consistently encountered mitochondria among the photogenic granules. They are for the most part concentrated in the outer differentiated layer of the photogenic cells.

The cytoplasm of the photogenic cells appears finely granular. We have not observed an endoplasmic reticulum within it. However, some of the smaller cytoplasmic granules may be related to certain of the components of this system.

Discussion

No effort will be made in this paper to review the extensive literature related to the histology of the light organs of fireflies. Buck (1948) has admirably treated this subject and the reader is referred to his paper for a critical analysis and references to the literature on firefly luminescence.

Because of its relatively small size and somewhat erratic staining, the tracheal end cell structure is not well understood. However, the end cell has long been considered as one of the basic structures involved in the mechanism of firefly luminescence. For example, Lund (1911) demonstrated that the tracheal end cell possessed remarkable powers for the reduction of osmic acid. He interpreted this to indicate that the end cell cytoplasm probably contained a specific reducing enzyme, reductase, thought to be important in bioluminescence. Based on these observations Lund states: "The photogenic process is localized in and adjacent to the cytoplasm of the photogenic cells and especially (as far as could be determined) where the cytoplasm of the tracheal end cell, and tracheole is applied to the photogenic cell."

Dahlgren (1917), in a superb series of papers on the histology of the light organs of animals, saw in the end cell of *Photinus consanguineus* three presumably different specialized bodies surrounding the tracheal twig. The proximal one is figured as a small darkly staining mass whose function is not discussed by Dahlgren. More distally is figured a series of rods of denser material which radiate from the region of the tracheal twig. At the very distal part of the end cell, at a point where the tracheal twig branches into tracheoles, a third body composed of a "rounded mass" of still denser material was encountered. Dahlgren (1917) conceived of these bodies as being composed of muscle-like fibers which served two possible functions: "First, to prevent the passage of air into the tracheole by compressing them; this would appear to be the function of the 'rounded mass'; second, to enlarge the terminal twig and end organ and thus fill it with a supply of air; this would seem to be a possible function of the radiating rods." Dahlgren was not aware that even the smallest tracheal elements possess well developed taenidia.

This view of end cell structure and function remains a prominent one to-day (Alexander, 1943). However, Buck (1948) in evaluating it states: "The location and morphology of the tracheal end-cell, and the fact that it is the chief site of reduction of inspired osmium tetroxide vapor, have engendered many suggestions that it functions in controlling the oxygen used in luminescence. However, tracheal end-cells, of which there appear to be at least two types, show no conclusive morphological evidence of being able to function in the way postulated."

The results of our study do not confirm the view of Dahlgren (1917) as to the structure of the tracheal end cell and tracheal end bulb or "rounded body." Instead of muscle-like fibers in the tracheal end cell we have found it to be composed of many villus-like structures that contain filamentous bodies which we interpret to be mitochondria. These mitochondria display transverse and longitudinally arranged lamellae comparable to those commonly observed in electron micrographs of other tissues (for example, see Palade, 1953 and Beams and Tahmisian, 1954). They also stain readily in Regaud's method which is often used for the demonstration of mitochondria.

Electron micrographs demonstrate that the end cell is limited by a well defined membrane. However, it is not clear whether or not the tracheal end cell should be considered a modified tracheal epithelial cell, or as some other cell type. We are inclined to accept the former view that it is a modified epithelial cell.

The origin of the villus-like bodies seems to be from a folding of the end cell membrane adjoining the intima of the tracheal twig. However, the details of how the villi are formed from the cell membrane cannot be completely established from our preparations. This modification of the cell membrane may conceivably be an adaptation for greatly increasing its internal surface area.

The "rounded body" of Dahlgren (1917), located at the distal end of the tracheal end cell, likewise was found in electron micrographs to be free of any form of fibers of the type thought of by Dahlgren. Instead, it contains small villus-like bodies radiating from the outer surface of the intima of the tracheal twig and tracheoles. These microvilli are not limited to the end bulb, but may extend in the same relative position for some distance along the tracheole. It is unknown whether or not they are characteristic of the tracheoles in the photogenetic layer or whether they may be a common structure of tracheoles. We can only report that examination of numerous sectioned tracheoles of a comparable size from the grasshopper did not reveal them. Their possible function is difficult to even postulate.

The end bulb, in addition to possessing microvilli, also contains many small mitochondria. The bulk of the mitochondria are located distally to the radiating microvilli.

The results reported here seem to confirm the view that the end cell bulb membrane and end bulb cytoplasm are continuous with that of the tracheoles for, at least, part of the distance traversed between the photogenic cells. However, the mitochondria except for a few isolated instances seem to be confined to the cytoplasm of the end bulb.

The differentiated outer cytoplasm of the photogenic cells is interesting because, firstly, it is free of photogenic granules and, secondly, because it is loaded with mitochondria. It is not clear why the mitochondria should be concentrated at the periphery of these cells, unless they are somehow involved in the photogenic reaction. In this connection Lund (1911) suggested that it is this layer which under certain conditions shows a high light intensity. Dahlgren (1917) postulated that this layer was impermeable to oxygen and served as a barrier for entrance of oxygen into the cells by routes other than that of the tracheoles.

We have not observed in electron micrographs the presence of nerves which might be considered to innervate the tracheal end cells. Since nerve tissue is relatively easy to identify in such preparations, the above evidence suggests, but does not prove, that nerves do not consistently innervate these cells.

Much discussion has occurred concerning mitochondrial structure (Palade, 1953; Sjöstrand, 1953; Glimstedt and Lagerstedt, 1953; Beams and Tahmisian, 1954; Powers, Ehret and Roth, 1955; Weinreb and Harman, 1955). In this material some of the mitochondria in the end cells show longitudinally arranged and others transversally arranged internal lamellae. If the internal lamellae are concerned primarily with the localization of enzymes it would seem to make little difference whether or not they are arranged transversely or longitudinally with respect to the long axes of the mitochondria.

Of interest in connection with the structure of the mitochondria within the villi of the tracheal end cell is that they seem to be composed of single relatively long bodies instead of groups of somewhat smaller ones.

It is beyond the scope of this paper to speculate regarding the possible physiological and biochemical role, if any, that the abundance of mitochondria in the tracheal end cell, end bulb and differentiated border of the photogenic cells may play in firefly luminescence. However, it would not seem amiss to point out that it is now established, due in large part to the work of Harvey (1952), that light in the firefly is due to the action of an enzyme, luciferase, upon its substrate, luciferin, which is located within the granules of the photogenic layer. In order for the reaction to occur in a test tube, the presence of water, oxygen, luciferase, luciferin, adenosine triphosphate (ATP), and Mg or Mn salts is essential (Harvey, 1952). It has also been established that the mitochondria act as the principal carriers of oxidative enzymes (Schneider, 1953). Accordingly, a correlation of the presence of large numbers of mitochondria within certain cells (muscle) with the expenditure of large amounts of metabolic energy by the same cells has been made (Levenbook, 1953). Might it not be that a similar relationship exists between the mitochondria of the tracheal end cell, end bulb, differentiated outer layer of photogenic cells and firefly luminescence as is thought to exist between mitochondria and the contraction of muscle? Furthermore, it has been established that, in addition to housing the important enzymes of the respiratory system, the mitochondria are rich in ATP. All of this suggests that they are the important energy-releasing structures within the cells. In this connection ATP has been found to be important in firefly luminescence (McElroy, 1947, 1951; Harvey, 1952, 1953). Gomori's (1941) alkaline phosphatase reaction seems to indicate that the tracheal end cell cytoplasm and the nuclei of the photogenic cells are relatively rich in this enzyme. Possibly the activity of the alkaline phosphatase is tied in with the mitochondria in the metabolic reaction involved in the production of firefly luminescence.

SUMMARY

1. The following structures were observed in the light microscope preparations of the firefly light organ: photogenic layer, reflector layer, photogenic granules, urate granules, transverse tracheae, tracheae, cylinders, tracheal twigs, tracheal end cells, tracheal end bulbs, tracheoles which extend between the photogenic cells, cuticle, differentiated outer layer of the photogenic cells, mitochondria in tracheal end cells and in differentiated outer layer of photogenic cells.

2. Urate granules were revealed in the electron micrographs to be of about the same density as the surrounding cytoplasm. Vacuoles which may be due to fixation artifact, or to a dissolution of material from within the granules, were observed. Filamentous mitochondria with pronounced transverse lamellae were observed lying between the granules of the urate cells.

3. Electron micrographs demonstrate, radiating from the centrally located region of the tracheal twig within the end cell, numerous cytoplasmic processes, or villi, that contain filamentous mitochondrial material. In some end cells the mitochondria within the villi show longitudinally oriented lamellae. In other end cells, the internal lamellae are arranged transversely to the long axes of the mitochondria. Thus, within the same type of cell the intra-mitochondrial structure is seen to vary.

4. The distal "rounded body" of Dahlgren, here referred to as the end bulb, is located at the distal part of the end cell. Within this body which surrounds the tracheal twig at the position where it branches into two tracheoles are four structures: First is the centrally located intima of the tracheal tube; radiating from near the outside surface of the tracheal intima are numerous microvilli about 2,000 Å in length; centrifugally to the radiating microvilli is a layer of relatively small yet numerous mitochondria; limiting the end bulb is the well developed end bulb membrane.

5. The membrane and cytoplasm of the end bulb are continuous with those of the tracheoles. Mitochondria, on the other hand, are mainly limited to the body of the end bulb.

6. The differentiated peripheral layer of the photogenic cells placed at the surfaces of the light organ and around the tracheal cylinders was found to be composed of a limiting membrane overlying a differentiated cortical cytoplasmic layer containing a large number of mitochondria.

7. The tracheoles extend between, and never into, the photogenic cells.

8. All elements of the tracheal system, including the smallest tracheoles, were observed to possess well developed taenidia.

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DESCRIPTION OF PLATES

Figures 1 to 7 are light microscope photographs; Figures 8 to 19 are electron micrographs. Key to figures is as follows:

C, cylinder
CMB, end bulb membrane
CME, end cell membrane
CMP, photogenic cell membrane
CU, cuticle
CY, cytoplasm
EC, tracheal end cell
DZ, differentiated zone of photogenic cytoplasm

EB, end bulb
EN, tracheal end cell nucleus
M, mitochondria
MB, mitochondria of end bulb
MM, microvilli
MP, mitochondria of photogenic layer
N, nucleus
PC, photogenic cell
PG, photogenic granule
PN, photogenic cell nucleus
RL, reflector layer
T, trachea
TE, tracheole
TV, transverse trachea
TW, tracheal twig
UG, urate granule
V, villus
VM, villus membrane
X, unidentified body

Scale on electron micrographs equals one micron.

IMMUNOLOGICAL STUDIES OF THE SERUM PROTEINS OF SOME REPTILES[1]

ELIAS COHEN[2, 3]

Department of Zoology, Rutgers University, New Brunswick, New Jersey

Serological studies have experimentally related the genetic nature of organisms to the biochemical similarities of their antigens and in turn to their taxonomy. A classic example is the work of Irwin and Cole (1937). The photronreflectometer quantitative precipitin technique as developed by Boyden and DeFalco (1943) has been successfully applied to Crustacea by Boyden (1936, 1939), by Leone (1949); to Insecta by Leone (1947a, 1947b); to Arachnida and other arthropods by Leone (1954); to Elasmobranchiata and Pisces by Gemeroy (1941, 1943); to Aves by DeFalco (1942), and to Mammalia by Boyden and Gemeroy (1950), Boyden and DeFalco (1943), and Moody *et al.* (1949).

Graham-Smith (1904) extended the mammalian and avian sera precipitin study by Nuttall (1904) to the sera of reptiles, amphibians, fish, and crustaceans. An extensive historical review of interordinal serological studies of reptiles was published by Erhardt (1930). A detailed analysis and translation of Erhardt's review is part of the doctoral thesis of Cohen (1952).

It is difficult to evaluate the results of the early precipitin studies of reptilian sera. None of the workers used comparable antigens or test methods. Important variables, as the temperature and time of incubation of reacting components, are not the same for each worker, nor are they always described. Their studies were not quantitative since absolute amounts of protein antigen were not determined;

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[1] Revision of a section of a thesis accepted as partially fulfilling the requirements for the Ph.D. degree, Rutgers University, New Brunswick, New Jersey.

[2] Present address: Biomedical Research Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma.

[3] Without the guidance of Dr. Alan Boyden and Dr. Ralph J. DeFalco, this study would not have been possible. Dr. Leslie Stauber, Dr. James Allison, Dr. Douglas Gemeroy, and Dr. Thurlow C. Nelson gave needed criticism and advice.

The Serological Museum contributed a good portion of the serum antigens used in this study or financed the shipment of such materials. Museum specimens used were donated by H. Volsoe and G. E. Pickford, the Galathea Expedition (1951); Dr. W. C. Osman Hill, the London Zoological Society; Dr. Leonard J. Goss, the New York Zoological Society; Mr. F. S. Wood, Jr., Marineland Studios; and Mr. Carl Shuster, Rutgers University.

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therefore, it was difficult to keep experimental conditions constant. Some unknown portions of the range of reaction between unknown amounts of antigen and antibody were accepted as serological evidence for taxonomic relationships. The careful quantitative photronreflectometer studies by Boyden and DeFalco (1943) have proved to be the most valid means for quantitatively indicating biochemical dissimilarity of heterologous and homologous antigens.

Snakes have attracted more serological study than have other reptiles. Kellaway and Williams (1931) used complement fixation, red cell agglutination, and precipitin tests to study some common Australian snakes. They concluded that the precipitin test did not differentiate closely allied genera in the family Colubridae. Absorbed precipitating sera, that were genera-specific, irregularly differentiated species. Complement fixations carried out with precipitating antisera were more specific and the results agreed with the zoological order of the genera studied. Red cell agglutination studies agreed with the zoological order, but were not any better than complement fixation tests.

Bond (1939) studied the hemagglutinins and hemagglutinogens of the bloods of some common North American snakes. No agglutination was observed between individuals of the same species nor between species of the same genera.

Wolfe (1939) included the unpublished studies of Bernice Cohen who found that an anti-chicken serum gave positive reactions with snapping turtle whole serum antigen. However, a rabbit anti-snapping turtle serum gave no reaction with bird, snake, or alligator sera antigens.

Most recently Kuwajima (1953) classified Formosan poisonous snakes by means of precipitin ring-tests and complement fixation reactions. It was difficult to get clear cut differentiation of the species studied, but a family specificity was noted by ring-test procedures. On the other hand, the complement fixation reaction gave a definite separation of snake species, but an even clearer demonstration of genus specificity.

A review of the immunological studies of venoms is outside of the scope of this paper. Cohen (1952) reviewed serological studies of snake venoms but found them to be of no practical value for the taxonomist.

Recently, a few outstanding papers have dealt with broad problems of the relationships and possible origins of snakes. Smith and Warner (1948) used a variation of the snake hyobranchium to delineate three distinct groups (superfamilies) of snakes and to derive a phylogeny of the snake families. Schmidt (1950) described modes of evolution of snakes based on their taxonomy. Since snakes have left few fossils and their genetics has hardly been explored, Schmidt emphasized that his phylogenetic tree of families was purely an inference. D'A. Bellairs and Underwood (1950) reviewed old and new literature of the origin of snakes and compared present-day relationships with those in the fossil record. Boyden and Noble (1933) indicated the fallacy of depicting phylogeny as a family tree in a single plane—such as Smith and Warner, Schmidt, D'A. Bellairs and Underwood have done. It has been Boyden's contention that a three-dimensional expression of current inter-relationships has more validity. Serological techniques lend themselves to quantitative expression of animal relationships.

In view of the dearth of quantitative serological studies of reptiles it was proposed to immunologically investigate the following categories:

(1) Inter-ordinal relationships of the Reptilia.
(2) Inter-familial relationships of the Ophidia.
(3) Inter-generic relationships of the Ophidia.
(4) Intra-generic relationships of the Ophidia.

MATERIALS AND METHODS

Blood sera used in these studies were obtained from the following species of reptiles:

Alligator mississipensis
Chelydra s. serpentina
Testudo (Aldabra)
Chrysemys elegans
Varanus nilaticus
Lapemis curtus
Naja n. naja
Coluber c. constrictor
Natrix s. sipedon
Crotalus v. viridis
Crotalus v. helleri
Crotalus r. ruber
Sistrurus m. barbouri
Sistrurus c. tergiminus
Agkistroden piscivorus
Agkistroden m. cupreus
Agkistroden m. mokasen

For the preparation of the antigens, most of the blood sera were collected in a similar manner. Snakes and turtles were decapitated and carcasses elevated so that the blood could be collected in test tubes. Clotted blood was allowed to retract overnight in a refrigerator. On the following day, the blood was centrifuged and clear serum above was decanted or drawn off by pipette. The serum was bottled in rubber-stoppered vials and Merthiolate (Eli Lilly and Co.) added to a final concentration of about 1 : 5000 or 1 : 10,000. Some specimens of sera were flown to the laboratory directly from California where they had been frozen in "dry ice." Other sera were supplied by the Serological Museum at Rutgers University.

The protein content of the sera was determined by several methods standardized on micro-Kjeldahl N data. The copper sulphate specific gravity technique of Phillips *et al.* (1950) was useful in the approximation of the amount of serum protein antigen in grams per cent. An Abbe Refractometer loaned by J. Allison, Director of the Bureau of Biological Research, Rutgers University, was used to construct a graph based on the refractive indices of dilutions of reptilian serum of a known (micro-Kjeldahl determined) protein concentration. Only a minute quantity of serum was necessary to determine the total protein concentration of a serum specimen once the standard curve was obtained. Valuable serum reagents were spared as well as hours of labor by using this technique. The Gornall *et al.* (1949) modification of the Kingsley (1940) biuret determination of total protein and albumin content of sera was also useful since it only requires small amounts of blood serum (0.25 to 0.5 ml.) to do complete A/G ratios. A Fisher Photometer (Fisher Scientific Co.) Model Serial No. 273 with a 525-millimicron filter was used for colorimetric biuret determinations. Data of much interest to the student of comparative biochemistry were obtained by Cohen (1954) by the simple methods described.

A total of 17 rabbits was used in the *preparation of the antisera.* Only one of these rabbits was presensitized by an intravenous injection of one ml. of the native serum via the marginal ear vein. All of the other rabbits were immunized by 3 or 4 daily one-ml. subcutaneous injections with a trial or final bleeding 7 days after the last injection. The site of injection was beneath the thin skin of the neck of the rabbit. Three powerful antisera, each prepared to a different native serum, were obtained by subcutaneous injections of 0.85% saline washes of the blood clots. The total protein content of the clot washes ranged from 0.7 to 1.0 gm. %. Antisera were Seitz-filtered or frozen and stored in a freezing compartment.

Antisera were produced to the blood sera of the following species:

Alligator mississipensis (Alligator)
Chelydra serpentina (Snapping turtle)
Lapemis curtus (Sea snake)
Coluber c. constrictor (Black racer)
Crotalus v. viridis (Prairie rattlesnake)
Agkistrodon piscivorus (Moccasin)
Sistrurus miliarius barbouri (Pygmy rattlesnake)
Naja n. naja (Indian cobra)
Varanus nilaticus (Monitor lizard)

The testing was executed by quantitative determinations of the turbidities that resulted when serial dilutions of antigen were mixed with a constant amount of antiserum, the Libby (1938) photronreflectometer technique. Standard procedures developed by Boyden and DeFalco (personal communication) were used to obtain and plot the data. Ring tests were occasionally used for a preliminary survey of heterologous reactions. Complete series of dilutions were not necessary, since it was found that a 1:2000 to 1:16,000 dilution (based on protein content) of the antigen would give a positive ring test if any precipitin reaction were to occur at all. Standard techniques were used for the ring tests as described by DeFalco (1941).

RESULTS OF IMMUNOLOGICAL COMPARISONS

1. Inter-ordinal relationships of Reptilia

An antiserum to the whole serum of *Alligator mississipensis* gave negative results by both ring tests and photronreflectometer determinations when tested with the sera of *Coluber c. constrictor* and *Varanus nilaticus,* respectively. However, a definite precipitin curve of 0.25% was obtained with the serum antigen of *Chelydra serpentina.* (See Table I.)

These results support the findings of Graham-Smith (1904). By crude ring tests he found a closeness of relationship between the Crocodilia and Chelonia. Anti-*Chelydra serpentina* tested with *Coluber c. constrictor* and with *Varanus nilaticus* gave negative results (no turbidity). Nevertheless, the curve area of this antiserum when tested with *Alligator* antigen was 6.30%. It was interesting to note that the living fossil, the Aldabra tortoise *Testudo* spp., showed almost the same per cent relationship to *Chelydra* as did the *Alligator*.

The rabbit anti-*Coluber* serum gave no reactions with *Chelydra* or *Alligator* antigen, but did give a 0.23% precipitin curve when tested with the antigen of the lizard, *Varanus nilaticus*.

Reciprocal tests with the antiserum to *Varanus* gave an 0.16% curve area when tested with the antigen of *Coluber*. However, no reactions were obtained with the turtle antigen, *Chelydra*, or with the serum of *Alligator*.

TABLE I

Summary of per cent relationships of species serum proteins tested

Per cent relationship of antigens	Antisera								
	Alligator missip.	*Chelydra serp.*	*Lapemis curtus*	*Coluber c. con.*	*Crotalus v. vir.*	*Agkistrodon pisciv.*	*Sistrurus m. barb.*	*Naja n. naja*	*Varanus nilaticus*
Alligator missip.	100.0	6.3		0.0					0.0
Chelydra serp.	0.25	100.0		0.0					0.0
Lapemis curtus			100.0	47.0	23.4			43.8	
Coluber c. const.		0.0		100.0	3.0			29.3	0.16
Crotalus v. viridis			3.9	46.3	100.0	65.9	40.6	19.1	
Agkistrodon pisciv.					54.2	100.0	27.5		
Sistrurus m. barb.					80.7	60.8	100.0		
Naja n. naja			17.0	32.8	5.3			100.0	
Testudo (Aldabra)		6.5							
Chrysemys elegans		22.1							
Sistrurus c. terg.					91.0	83.9			
Crotalus r. ruber					87.5	69.2			
Crotalus v. helleri					90.0				
Agkistrodon m. cupreus						55.7			
Agkistrodon m. mokasen						86.1			
Varanus nilaticus	0.0	0.0		0.2					
Natrix s. sipedon					4.3				

A different set of rabbits might have given somewhat different values. However, it is significant that the order of placement would probably still have been the same. From the data obtained by the photronreflectometer precipitin curves, the alligator and turtle are closer to each other than they are to the snake and lizard. On the other hand, the snake and lizard are serologically closer to each other than they are to the alligator and turtle. These observations certainly verify the interpretations based on morphology alone.

2. *Inter-familial relationships of Ophidia*

In this study, comparisons were made of carefully selected representatives of four major families of snakes. (See Table I.)

When anti-*Lapemis* (sea snake) serum was tested with *Crotalus, Naja,* and *Coluber* antigens, its precipitin reactions gave this series of relationships: *Lapemis-Naja-Coluber-Crotalus*.

Reciprocal tests with each of the other three antigens (families) definitely placed *Lapemis* and *Naja* closer together serologically than they are to the other two families. From the results, it is apparent that *Coluber* is serologically more like the cobra and sea snake than like the rattlesnake (*Crotalus*). This supports the morphological data. Smith and Warner (1948), Schmidt (1950), and d'A. Bellairs and Underwood (1950) place the colubrine snakes, as *Coluber*, close to the cobras and sea snakes. The term "supports" is preferred because serological data cannot be construed to prove or disprove phylogeny. Instead, comparative serology provides objective quantitative data concerned with the biochemical natures of the existing organisms compared.

3. *Inter-generic relationships of Ophidia*

In one family, the Crotalidae, genus *Sistrurus*, the pygmy rattlesnakes, is obviously unlike any of the other rattlesnakes, genus *Crotalus*. Herpetologists have separated *Sistrurus* from *Crotalus* primarily on these observations.

(a) *Sistrurus* has 9 symmetrical head plates, *Crotalus* has a head covered with small scales.

(b) *Sistrurus* has genital characters that are different from those of *Crotalus*.

Cope (1900) pointed out the similarities between the genital characters of *Sistrurus* and *Agkistrodon*, the moccasins. Gloyd (1940) believes that *Sistrurus* cannot logically be derived from any present species or group within the genus *Crotalus*. Amaral (1929) proposed that the *triseriatus* group of *Crotalus* was closely related to *Sistrurus*. He did this because *Crotalus triseriatus* possesses enlarged head scales, that resemble shields. Gloyd did not concur with Amaral, because of the differences of head scale and genital characters between the two groups of snakes.

Since additional criteria, or discernible characters, might contribute towards a broader perspective of the relationships of *Sistrurus*, serological comparisons were made of representatives of the three genera.

Adult pygmy rattlesnakes are usually no longer than 15 inches. Therefore, the blood sera of four adult specimens had to be pooled to yield enough antigen for this study. The antisera to *Agkistrodon piscivorus* and to *Crotalus v. viridis* were also produced by pooled whole serum antigens.

A doubling serial dilution of *Sistrurus* antigen was prepared over a per cent protein dilution range of 1 : 500 to 1 : 512,000. When tested with the rabbit anti-*Sistrurus* serum, a photronreflectometer precipitin curve was obtained with a curve area of 229 turbidity units.

Rabbit anti-*Sistrurus* serum × *Crotalus* gave a turbidity curve area of 40.61% of that of the homologous curve.

Rabbit anti-*Sistrurus* serum × *Agkistrodon*, however, gave a curve area 27.5% of that of the homologous curve.

Reciprocal tests gave the following % relationships based on curve areas:

Rabbit anti-*Agkistrodon* serum × *Agkistrodon* antigen = 100%
Rabbit anti-*Agkistrodon* serum × *Crotalus* antigen = 65%
Rabbit anti-*Agkistrodon* serum × *Sistrurus* antigen = 60.80%
Rabbit anti-*Crotalus* serum × *Crotalus* antigen = 100%
Rabbit anti-*Crotalus* serum × *Sistrurus* antigen = 80.78%
Rabbit anti-*Crotalus* serum × *Agkistrodon* antigen = 54.20%

It is known from other serological studies (Boyden, 1942) that although the percentage may vary in reciprocal tests, the zoological order of the series still remains the same. Examination of the per cent relationships reveals that the serum proteins of the *Sistrurus* species used are closer to those of *Crotalus v. viridis* than to those of *Agkistrodon piscivorus.*

As limited as these data are, they are as suggestive and provocative as any systematist's speculations about the phylogeny of the genus *Sistrurus*. Serum proteins are known to be relatively conservative characters. The pygmy rattlesnakes are serologically closer to the "true" rattlesnakes (*Crotalus*) than to the moccasins, in agreement with modern taxonomy.

It is certainly subjective and difficult to decide how "typical" a typical representative of a group of vertebrates is. The most valid procedure is to compare all members of a group, or as many as possible, by appropriate reciprocal tests.

Only a small amount of serum of a single specimen of *Sistrurus catenatus tergeminus* was available for comparisons. It was used as a test antigen with antisera to both *Agkistrodon* and *Crotalus.*

The available reciprocal results are tabulated as per cent relationship values in Table I. The data suggest that the massasauga, *Sistrurus c. tergeminus,* is closer to the rattlesnake (*Crotalus*) than to the moccasin (*Agkistrodon*). Yet, on the other hand, the reaction with antiserum to moccasin superficially conveys an opposite impression. This discrepancy can be resolved, since the massasauga is quite a distinct group within the genus *Sistrurus*. Possible tentative explanations, pending further serological analysis, are:

(a) The massasauga is more closely related to the rattlesnake than to the moccasin. The geometrical figure representing their inter-relationships might then be an obtuse triangle.

(b) There is a possibility that the antisera contained very disproportionate amounts of anti-albumins and anti-globulins.

A more intensive serological study of other species of *Crotalus, Agkistrodon,* and *Sistrurus* would be necessary to completely verify the above results.

4. Intra-generic relationships of Ophidia

Monographs have been written on the morphology of many genera and their constituent species. To treat a single genus of snakes adequately would require a complete series of all the species of that genus. Fortunately a number of rattlesnake and moccasin species were available for serological study.

(a) Antisera to the pooled whole sera of *Crotalus v. viridis* when tested gave the following per cent relationship values:

Species	Per cent relationship values
Crotalus v. viridis	100.0
Crotalus v. heller	90.0
Crotalus r. ruber	87.0

(b) Antisera to *Agkistrodon piscivorus* when tested with antigens gave the following per cent relationship values:

Species	Per cent relationship values
Agkistrodon piscivorus	100.0
Agkistrodon mokasen cupreus (Kansas copperhead)	86.0
Agkistrodon mokasen mokasen (Maryland copperhead)	55.0

It would not be valid to draw any broad conclusions regarding the serological relationships of all the species, since antisera were prepared to only two of the species compared. Nevertheless, the data suggest that the species of rattlesnakes are more closely related to each other than are the species of moccasins studied.

There is evidence in herpetological literature to support this supposition. Two of the six forms of *Sistrurus* and twenty-four of the thirty-nine of *Crotalus* occupy the Mexican Plateau or nearby areas (Gloyd, 1940).

However, the genus *Agkistrodon* and a related genus *Trimeresurus* are found discontinuously distributed in southeastern Asia, the East Indian region, one part of eastern Europe, parts of southern Mexico, Central and South America, as well as the United States (Schmidt and Davis, 1941).

The spotty distribution of the genus *Agkistrodon* implies geographic isolation. Isolation, particularly of small sexually reproducing populations, has been known to accentuate variation. In that case, the moccasin species may be more distinct genetically from each other than are the rattlesnakes.

No decisive aid has come from cytological studies of snakes. An inspection of the atlas of chromosome numbers by Makino (1951) reveals slight variation between *Crotalus* and related genera.

Fossil evidence is sparse, consisting mainly of isolated vertebrae, ribs, fangs, and lower jaw bones. *Crotalus* was widespread in North America during Pleistocene times. From the fossil record it has been supposed that the rattlesnakes originated at a time more remote than the Pliocene, or, at the latest, before the Pleistocene. Crotalid-like remains have been found which cannot be classified as *Agkistrodon, Sistrurus,* or *Crotalus* (Gilmore, 1938).

From the above discussion it can be seen that serological comparisons have much to offer the herpetologist-systematist. Data from such study provide relative *quantitative* and *objective* criteria supplementary to available systematics. Where the serological relationships do not agree with generally accepted morphological relationships, then a thorough re-examination should be made of both.

SUMMARY

1. Convenient methods of obtaining and preparing the serum antigens of reptiles are described. Antisera are prepared principally by a subcutaneous mode of injection. Precipitin testing is almost exclusively by the quantitative photron-reflectometer procedure of Boyden and DeFalco. Ring tests are used to verify the weakest heterologous reactions.

2. Quantitative investigation of the systematic serology of common reptiles corroborated the findings of Graham-Smith. The alligator was serologically closer to the turtle (*Chelydra*) than to either lizard (*Varanus*) or snake (*Coluber*). Quantitative serological comparisons revealed that *Lapemis* (sea snake) and *Naja* (cobra) were closer to each other than to *Coluber* (racer) or *Crotalus* (rattlesnake). The pygmy rattlesnake, *Sistrurus,* was found to be serologically closer to *Crotalus* ("true" rattlesnake) than to *Agkistrodon* (moccasin), some of whose morphological features it shares. From serological study of available material, it was suggested that the rattlesnakes are more closely interrelated than are the moccasins. Such information compares favorably with the fact that the moccasins are discontinuously distributed geographically, whereas the rattlesnakes are not.

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MORTALITY OF OLYMPIA OYSTERS AT LOW TEMPERATURES

HARRY C. DAVIS

U. S. Fish and Wildlife Service, Milford, Connecticut

The ability, or inability, of organisms to become acclimated to a new environment becomes economically important when, for example, it is desired to increase the number of oysters or clams in a given area by transplanting seed oysters or clams from a different geographical area. It is also of importance when trying to establish oysters or clams in areas where they do not now exist.

In the fall of 1948 a shipment of Olympia oysters, *Ostrea lurida,* was brought to Milford, Connecticut from Puget Sound for experiments with their larvae. This shipment was divided into two groups. The group that was kept suspended in Milford Harbor, which freezes in winter, suffered 100 per cent mortality.

The second group kept in the laboratory, in running sea water at temperatures above 10.0° C., survived the winter and later provided the larvae for our experiments. The larvae were reared to metamorphosis and the young oysters reached a size of 3.5 to 3.7 cm. some 70 days later (Davis, 1949). Since oysters that set in April or May become sexually mature by the following spring, if protected during the winter, we were able to maintain our experimental stock by rearing a new generation every year.

Records, from the fall of 1948 through the winter of 1954–55, show that in Milford Harbor the water temperature, quite regularly, remains below 10.0° C. from some time in November until the end of April, and ranges from 5.0° C. to −1.0° C. from mid-December until the end of March. To prevent a complete loss of our Olympia oysters, through winter killing, it was necessary to bring some of them into the laboratory between mid-December and mid-January each year.

During the winter of 1948–49 the Olympia oysters that suffered 100 per cent mortality had been below the ice, in wire baskets suspended from floats. Those kept in Milford Harbor during the winters of 1949–50, 1950–51, and 1951–52, were under similar conditions and, while detailed records were not maintained, only those kept in the laboratory throughout the winter survived. In 1952–53 we had one of our outdoor tanks covered to prevent freezing. Although the temperature in this tank was approximately the same as in Milford Harbor, no ice formed. The Olympia oysters left in this tank all winter, nevertheless, showed 100 per cent mortality (Table I).

The following winter (1953–54) 101 Olympia oysters of the 1952 year-class, and 160 from the 1953 year-class, were left in hatchery-type tanks of running sea water in our unheated dockhouse throughout the winter where, again, no ice formed. An additional 834 from the 1953 year-class were brought into the laboratory about mid-January. One group of 528 of the latter was kept in unheated running sea water at a temperature that rarely rose above 5.0° to 10.0° and occasionally dropped to 3.0° or 4.0° C., while the remaining 306 were kept in running sea water at about 10.0° to 12.0° C. The group kept at 10.0° to 12.0° C. suffered appreciably less mortality than the other group kept in the laboratory at the lower temperature, and the latter group in turn suffered less mortality than those left in the dockhouse all winter (Table I). It was suspected, however, that much of the mortality in both laboratory groups was a continuation of the winter mortality that was already apparent in the dockhouse before these oysters were brought in.

During the winter of 1954–55 Olympia oysters of the 1954 year-class were suspended from the dock well below the ice line. About mid-December, before any mortality was apparent, 191 of these oysters were brought into the laboratory and kept at about 12.0° C., while the remaining 625 Olympia oysters were left suspended from the dock all winter. When these two groups of oysters were examined March 30, 1955, only seven, or 3.6 per cent, of those at 12.0° C. were dead and at least two had probably died of shell injuries. The 625 oysters left suspended from the dock, however, were all dead by March 30.

TABLE I

Winter mortality of O. lurida at Milford Laboratory under different conditions

Year	Place oysters were kept	Number of oysters	Per cent mortality
1948–49	Suspended from float; under ice	Several hundred	100.0
1949–50 1950–51 1951–52	Suspended from float; under ice	Several hundred	100.0
1952–53	Baskets in covered outdoor tank; lowest temp. −0.7° C.	Several hundred	100.0
1953–54	Dockhouse; lowest temp. −0.7° C.	1952 set—101 1953 set—160	100.0 100.0
	Laboratory; 5.0–10.0° C. or lower	1953 set—528	86.74
	Laboratory; 12.0–13.0° C.	1953 set—306	60.50
1954–55	Suspended from dock; below ice	1954 set—625	100.0
	Laboratory; 12.0–13.0° C.	1954 set—191	3.6

Since death usually occurs only after a month or more of exposure to low temperature, it might be assumed that it was the result of a depletion of stored food brought about by the prolonged period of water temperatures below their feeding temperature. This does not appear to be the explanation, however, since a majority of the dying oysters still contained a good reserve of glycogen.

Loosanoff and Nomejko (1951) have found that even oysters of the same species, *Crassostrea virginica,* as our Long Island Sound oysters, namely, oysters from North Carolina and Florida, suffered 87.0 per cent and 97.0 per cent winter mortality when kept in Milford Harbor. They also found that *C. virginica* from Virginia and also from nearby New Jersey did not become completely acclimated, even after two years in Long Island Sound, as shown by their failure to spawn.

Conversely, Loosanoff (1951, 1955) has shown that the European oyster, *Ostrea edulis,* a larviparous oyster closely related to *O. lurida* and belonging to the same genus, could not only survive in American waters but could reproduce as far north as Maine.

Imai *et al.* (1954) reported that seed oysters, produced from *O. lurida* imported to Japan, "all died of freezing" during the winter of 1950 at Mangoku-Ura. In 1954 they had seed oysters at Saroma, Usu and Onagawa Bays and state that the mortality of *O. lurida* was nearly twice as high as for the native Japanese oyster at each bed. Moreover, their data seem to indicate that the mortality of *O. lurida* increased progressively from Onagawa, in the south, to Saroma, their most northern station. Thus, their observations closely parallel our 1953–54 data which showed progressively increasing mortality as the temperature at which *O. lurida* was kept was decreased.

Our observations indicate that the winter mortality of Olympia oysters in Milford Harbor results from an inability to withstand prolonged exposure to low temperatures, and that this inability is inherited. Our breeding stock was selected each year, to the extent that the oysters used to rear a new generation were brought into the laboratory only after the least resistant had already succumbed to the low water temperature in the harbor. Nevertheless, even after five generations at Milford, Olympia oysters have still not become acclimated to the prolonged low temperatures of our winters.

The author wishes to express his appreciation to Dr. V. L. Loosanoff, Director of Milford Laboratory, for his continued interest and counsel in this project.

SUMMARY

1. Olympia oysters, kept throughout the winter in Milford Harbor, showed 100 per cent mortality.

2. The mortality of these oysters increased progressively as the temperature at which they were kept was progressively decreased.

3. Death usually occurred after a month or more of exposure to low temperatures.

4. Mortality did not appear to be due to tissue starvation, since many of the dying oysters still contained a good reserve of glycogen.

5. Although our breeding stock was selected, each year, after the least resistant oysters had already succumbed, Olympia oysters, even after five generations at Milford, had still not become acclimated to our prolonged period of low temperatures.

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BODY WEIGHT, CELL SURFACE, AND METABOLIC RATE IN ANURAN AMPHIBIA[1]

JOHN DAVISON

Department of Zoology, University of Minnesota, Minneapolis, Minn.[2]

It can be regarded as a general phenomenon in the animal kingdom that small animals exhibit a higher standard metabolic rate than related animals of greater body weight (Zeuthen, 1949). Rubner (1883) considered the homeotherm as a body at constant elevated temperature, losing heat from the exterior, and consequently exhibiting a basal metabolic rate directly proportional to the external body surface.

That is:

Metabolism/time = $k\ W^{\frac{2}{3}}$ (surface law of metabolism)

Since poikilothermic animals also exhibit size-dependent metabolic rate functions, and since they do not maintain a constant temperature, the considerations of Rubner do not apply to the general problem. One must, then, consider other size-dependent factors which may determine the metabolic rate differences between large and small animals.

One such size-dependent factor may reside in the cellular organization of the animal. If the metabolic performance of the animal is conditioned by reaction systems localized at cell surfaces, then it is reasonable at the outset to expect surface-dependent metabolic activities similar to those observed in homeotherms and poikilotherms alike. The cell surface is presumably directly concerned with such energy requiring processes as the maintenance of ionic disequilibria and the entrance and exit of specific materials (Rothstein, 1954).

Plotting the findings of several authors, Zeuthen (1953) has found a value of approximately ⅔ (0.7) for the exponent in the above equation, for the size range of unicellular organisms (bacteria to the large ameba *Pelomyxa*). In the protista, of course, external body surface and cell surface are identical.

One can extend the consideration of cell surfaces to the metazoa as follows:

Consider a hypothetical animal which grows isogonically by means of cell enlargement. External body surface and internal cell surface *each* vary directly as the body weight (W) raised to the ⅔ power. The other obvious means of growth is by the formation of additional cells of fixed size. In this case, total cell surface varies as W^1, while external body surface varies as $W^{\frac{2}{3}}$. If metabolic rate were strictly a function of cell surface, one *a priori* would expect metabolic rate to vary directly as the body weight raised to some power between ⅔ and 1 in accord with the manner in which the organism increases in mass.

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[1] This paper represents in part a thesis in Zoology presented to the faculty of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address: Department of Zoology, Washington University, St. Louis, Missouri.

With regard to hypothetical interspecific comparisons, one might anticipate a value of the exponent between ⅔ and 1 since larger animals might have (a) the same number of cells, (b) larger and more numerous cells, or (c) more cells of the same size.

Table I, compiled from various sources, indicates the approximate value of the exponent e in the allometric expression:

Metabolism/time $= k\ W^e$.

As the table indicates, e as a generalization does in fact vary between ⅔ and 1.

For vertebrates, with the exception of fish, the exponent is near ⅔. Following birth in mammals few or no additional skeletal muscle fibers are formed (Maximow and Bloom, 1948). It follows that subsequent growth of these elements is by enlargement. Trout skeletal muscle has been demonstrated to grow partly by the enlargement of fibers once formed, and partly by the addition of new fibers from myoblast cells (Gray, 1931). A value of e greater than ⅔ would be in accord with this manner of growth.

TABLE I

Group	e*	Source
Protista	0.7	Zeuthen, 1953
Vermes	0.9	Zeuthen, 1949
Crustacea		
Pugettia	0.798	Weymouth *et al.*, 1944
Several	0.8	Weymouth *et al.*, 1944
Insects	0.6–1.0	Zeuthen, 1949
Fish	0.7–0.8	Zeuthen, 1949
Fish	0.67	Rubner, 1924
Frogs		
R. esculenta	0.67	Rubner, 1924
Reptiles		
Lizards	0.67	Zeuthen, 1949
Reptiles	0.67	Benedict, 1932
Homeotherms	0.73–0.75	Brody, 1945 Benedict, 1932 Kleiber, 1947

* e represents the approximate value of the exponent in the allometric expression: metabolism/time $= k\ W^e$.

The objectives of the present study may be stated:

(1) To determine the standard metabolic rates of a reasonably homogeneous group of poikilothermic vertebrates (frogs), with body weight and species the major variables.

(2) To examine this standard metabolism as a function of the cell surface of homologous skeletal muscles in the same animals.

MATERIALS AND METHODS

Animals

The animals used in this study constitute an approximately geometric series of adult body weights as follows: *Pseudacris nigrita* ca. 1.0 gm., *Hyla versicolor* and *Hyla cinerea* ca. 4.5 gm., *Rana sylvatica* ca. 6.0 gm., *Rana pipiens* and *Rana clamitans* ca. 35.0 gm., *Rana catesbiana* ca. 350 gm.

R. pipiens and *R. catesbiana* were supplied from a local dealer. *H. cinerea* were supplied by General Biological Supply, Chicago, Illinois. *P. nigrita, R. clamitans,* immature *R. pipiens* and adult *R. sylvatica* were collected by the author from ponds near Minneapolis. *R. pipiens* were collected as they completed metamorphosis in early July, and *P. nigrita* tadpoles were collected in terminal stages and allowed to complete metamorphosis in the laboratory. Small *R. sylvatica* metamorphosed from tadpoles reared from the egg on a diet of oatmeal and algae.

Adult animals were maintained without food in aquaria for a few days to two weeks following field collection to allow for cessation of digestive activity and for acclimatization to room temperature (ca. 24° C.). The animals typically became quiescent following maintenance in the laboratory and were more likely to remain at rest in the respirometer than were animals fresh from the field. Metamorphosing animals were maintained without terrestrial food and respiratory rate measurements were made within one week following the completion of metamorphosis (complete resorption of the tail).

Approximately equal numbers of each sex were used throughout.

Determination of respiratory rate

The instruments used were similar in design to those used by Flemister and Flemister (1951). These were closed system volumetric respirometers consisting simply of two vessels connected by a manometer, one vessel containing the animal in saturated humidity, with the second vessel acting as a thermobarometer, serving to make the instrument differential. A beaker in the animal chamber containing the absorbent (10% KOH) was surrounded by plastic screening to prevent injury to the animal. The total volume of the animal chamber could be varied by means of a calibrated syringe. After equilibration in the water bath, both vessels were clamped off from the outside and the pressure maintained equal in the two vessels by varying the total volume of the animal chamber with the syringe. The measured decrease in volume of the animal chamber was thus equal to the oxygen consumed. Such a simple system is possible since frog O_2 consumption is independent of pO_2 down to about 45 mm. Hg (Jordan, 1929). A saturated humidity also constitutes a favorable environment for amphibian material.

The respirometers were of three principal sizes with animal chamber volumes of ca. 50 cc., 500 cc., and 4000 cc. The latter instrument, used for *R. catesbeiana* and large *R. pipiens,* was constructed from a desiccator jar with a three-liter reagent bottle serving as a thermobarometer. A 50-cc. syringe was mounted outside the bath. Aquaria served as water baths. A fixed mercury thermostat, with a relay box heater and stirrer, gave temperature control to 25 ± 0.1° C. Following thermal equilibration, the O_2 uptake was followed with the animal at rest for 40 to 300 minutes.

The majority of experiments were carried out with single animals in the respirometer, due to the small probability of two or more animals remaining at rest for extended periods of time. However, successful experiments in which the number of animals varied compared favorably with determinations with single individuals. Similarly, the same animals measured in different instruments gave comparable values for O_2 uptake. With the animals fasting and at rest for extended periods of time, the metabolic rate of spring animals was not found to differ from that of winter animals. The seasonal differences reported in the literature (Oppenheimer, 1927) would seem largely to be due to differences in activity and general irritability. The data reported here are for the conditions of essentially no overt activity. A frog placed in the respirometer will usually come to rest after a few minutes of exploratory activity. An additional 20 to 30 minutes are often required before O_2 uptake becomes linear with respect to time. A steady rhythmic buccopharyngeal oscillation served as an index to a standard state. By means of a tenth-second stopwatch, ranid buccopharyngeal frequencies were determined during the time course of the O_2 uptake determination. Hylid buccopharyngeal oscillations typically became intermittent or ceased entirely when the animal became settled. The animals were weighed to two significant figures either just before or just after the respiratory rate determination. The respiratory rate is given as cc. O_2/hr./gm. live weight.

Determination of muscle cell surface

The animals were pithed, weighed and placed on freezer paper with the hind legs in a semi-flexed position. After freezing in the deep freeze, the lower leg was transected through the belly of the gastrocnemius, and a thin section of the gastrocnemius cut free-hand with a single-edge razor. The section was placed on a slide in a few drops of amphibian Ringer and observed under the compound microscope. By means of camera lucida the outlines of about 50 cells were traced on standard weight cards. By weighing the individual cell tracings and a known projected area, it was possible to obtain the cross-sectional areas of the individual cells from the proportional weights. From the cross-sectional area a circumference was calculated assuming the fiber is circular in cross-section. For unit length of long cylinders, volume becomes proportional to cross-sectional area, and surface to circumference. Summing the individual areas and circumferences, it was possible to express surface as $cm.^2$ surface/$cm.^3$ fibers, this quantity being here defined as the surface concentration of the muscle. Use of the same technique indicated extrafibral spaces of 20–25% for all species examined.

RESULTS

Respiratory rate

Figure 1 represents the relationship between metabolic rate and body weight. Each point represents a single determination of the resting rate of O_2 consumption. For individual species, the slopes of log (cc. O_2/hr./gm.) vs. log (wt. gms.) were found to approximate $-\frac{1}{3}$, indicating that the surface law holds within limited groups.[3] Figure 1 represents the double-log plots converted into semi-logarithmic form. The three largest species (*R. catesbiana*, *R. pipiens*, and *R. clamitans*) are represented by one plot. The hybrid species (*H. versicolor*, *H. cinerea*, and *P. nigrita*) are represented by a second plot. *R. sylvatica*, intermediate in adult weight between the other groups, is represented by a third plot of metabolic rate vs. body weight. Thus, three separate values of k in the surface law equation correspond to the three groups of data.

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[3] cc. O_2/hr. $= k\ W^{\frac{2}{3}}$ (surface law): dividing each side by grams this becomes: cc. O_2/hr./gm. $= k\ W^{-\frac{1}{3}}$: and taking logarithms of both sides: log(cc. O_2/hr./gm. $= -\frac{1}{3}$ log W + log k.

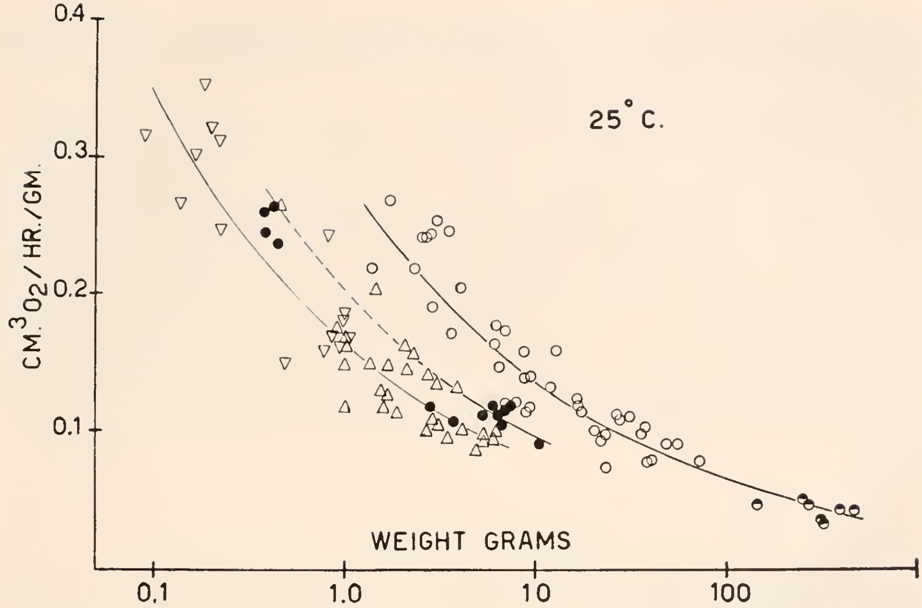

Figure 1. Resting metabolic rate vs. body weight. (○ *R. pipiens* and *R. clamitans;* ◓ *R. catesbiana;* ● *R. sylvatica;* △ *H. cinerea* and *H. versicolor;* ▽ *P. nigrita.*) Three equations of the form cc. O_2/hr./gm. $= k\ W^{-1/3}$ describe the data, k being a group constant. The dotted line in the *R. sylvatica* plot indicates that metabolic rate measurements were limited to newly metamorphosed and adult animals.

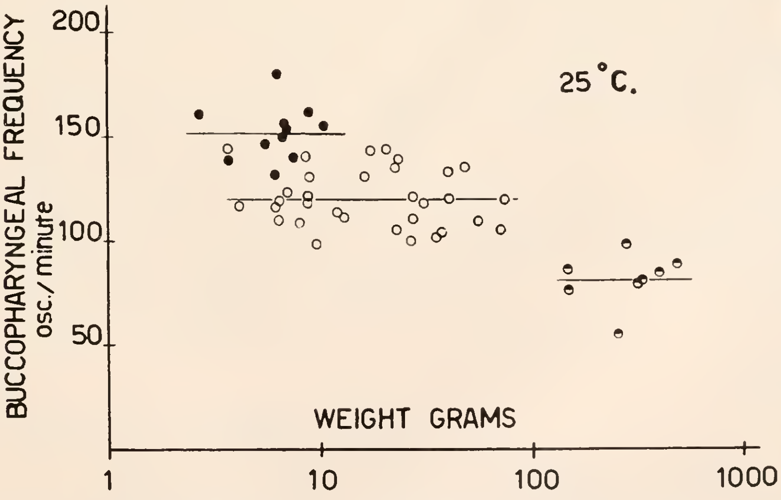

Figure 2. Buccopharyngeal frequency vs. body weight in the Rana species. (○ *R. pipiens* and *R. clamitans;* ◓ *R. catesbiana;* ● *R. sylvatica.*) A horizontal line has been drawn through the mean for each species. (Frequency $= k\ W^{\circ}$, k being a species constant.)

A precise determination of the numerical value of the exponent is difficult since a large geometric size range is not available within the weight range of each group. However, within the limits of the groups mentioned, the surface law equation would seem adequately to describe the data.

Buccopharyngeal frequency as a function of body weight appears in Figure 2. Each point represents the mean of several determinations of buccopharyngeal frequency obtained during the time course of the respiratory rate determinations. For the weight range from 4 to 72 grams, there is no indication that frequency is size-dependent in *R. pipiens* and *R. clamitans.* Since buccopharyngeal frequency seems to be a species constant, itself independent of body weight, the horizontal lines in Figure 2 have been drawn through the mean frequency for each of the ranid species. *R. clamitans* is assumed to belong in the same group with *R. pipiens.* Buccopharyngeal frequencies observed in newly metamorphosed animals were typically irregular and slightly higher than in somewhat larger juvenile animals. The data represented in Figure 2 are for frogs exclusive of the newly metamorphosed animals.

Muscle cell surface

Gastrocnemius surface concentrations are represented as a function of body weight in Figure 3. Each point represents the surface concentration calculated from an analysis of about 50 cells. Plotting log ($cm.^2$ surface/$cm.^3$ fibers) vs. log (wt. gms.), linear plots were obtained with slopes approximating $-\frac{1}{3}$ for each species. Figure 3 represents the double-log plots converted into semi-logarithmic form. Each species is then represented by the equation $cm.^2/cm.^3 = k\,W^{-\frac{1}{3}}$, k being

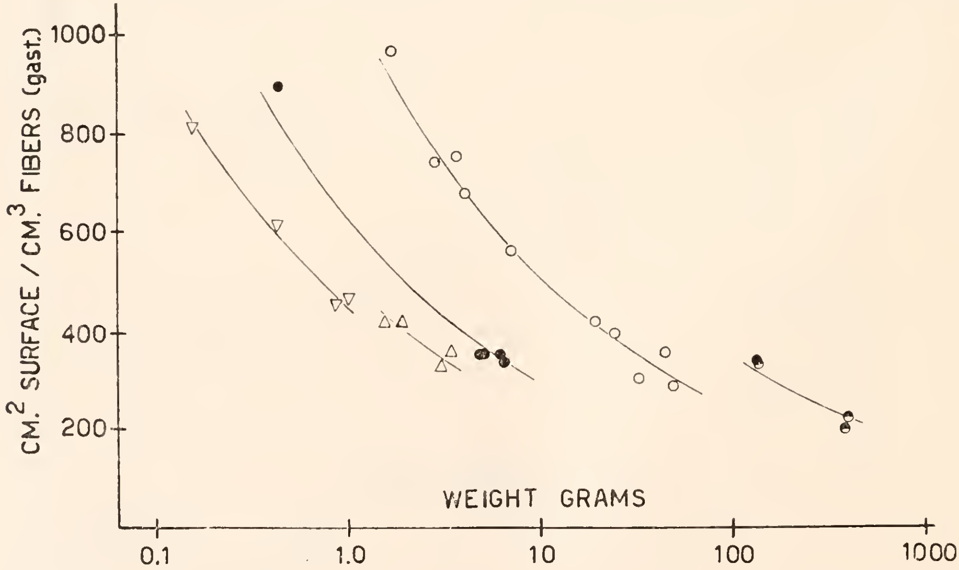

FIGURE 3. Gastrocnemius surface concentration vs. body weight. (○ *R. pipiens* and *R. clamitans;* ◓ *R. catesbiana;* ● *R. sylvatica;* △ *H. cinerea;* ▽ *P. nigrita.*) Each species is represented by the equation $cm.^2/cm.^3 = k\,W^{-\frac{1}{3}}$, k being a species constant.

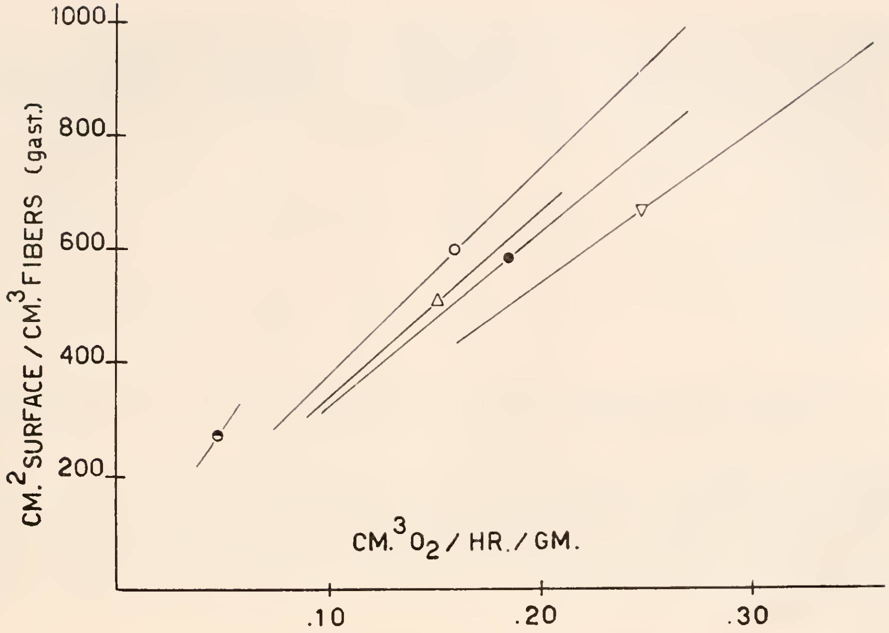

FIGURE 4. Resting metabolic rate vs. gastrocnemius surface concentration. The figure represents the metabolic rate equations plotted vs. the surface concentration equations over the weight range for which metabolic rate determinations were made. Symbols are as in Figure 3.

a species constant. *R. clamitans* is assumed to belong to the same plot with *R. pipiens*. All sections examined indicated a bimodal frequency distribution of fiber cross-sectional areas, with about equal numbers of large and small fibers.

Since muscle surface concentration varies directly as $W^{-\frac{1}{3}}$, it would seem that growth of the muscle is accomplished strictly by enlargement of fibers present at the completion of metamorphosis. Preliminary inspection indicated that the findings reported here for gastrocnemii represent generally the quantitative relationships of other homologous muscles in the same animals.

Since both metabolic rate and muscle surface concentration vary directly as $W^{-\frac{1}{3}}$, metabolic rate and surface concentration will be directly proportional to each other for each species considered separately. Rearranging the metabolic rate and surface concentration equations, they may be written:

$$\frac{\text{cc. O}_2\text{/hr./gm.}}{k} = W^{-\frac{1}{3}} = \frac{\text{cm.}^2\text{/cm.}^3\text{ fibers}}{k'}$$

and eliminating the common term:

$$\text{cc. O}_2\text{/hr./gm.} = \frac{k}{k'}\text{ cm.}^2\text{/cm.}^3\text{ fibers.}$$

It is thus possible to eliminate weight as a graphic variable and examine directly the relationship between metabolic rate and muscle surface concentration. Figure 4

TABLE II

Species and adult weight, gms.	cc. O_2/hr./gm. 25° C. (S.T.P.)	cm^2. surface/cm.^{3} fibers (gast.)	Buccopharyngeal frequency (osc./min.)
R. catesbiana 350 gms.	0.043	230	81
R. pipiens and *R. clamitans* 35 gms.	0.093	340	120
R. sylvatica 6.0 gms.	0.108	345	151
H. cinerea and *H. versicolor** 4.5 gms.	0.102	325	—
P. nigrita 1.0 gm.	0.162	450	—

* No analysis of muscle surface concentration appears for *H. versicolor*. Inspection of sections indicated similarity with *H. cinerea* of comparable body weight.

represents the equations represented in Figures 1 and 3 plotted against one another over the weight range for which metabolic rate determinations were made.

The tabular information for metabolic rate, buccopharyngeal frequency, and gastrocnemius surface concentration is indicated in Table II. O_2 volumes are reduced to 760 mm. Hg and 0° C.

DISCUSSION

Theoretical treatment of the data

The finding that the surface law equation describes the intraspecific relationship between metabolic rate and body weight is supported by the earlier finding of Rubner (1924) with *R. esculenta*.

It may be seen from Figure 4 that although metabolic rate and muscle surface concentration are directly proportional intra-specifically, they are not so interspecifically. However, those animals with similar adult metabolic rates (*H. cinerea, R. sylvatica*, and *R. pipiens*) exhibit similar adult muscle surface concentrations (Table II).

Buccopharyngeal frequency in the ranid species is apparently a species constant (Fig. 2), independent of body weight. Restricting the considerations to the ranid species, those functions which are continuous intra-specifically (buccopharyngeal frequency and muscle surface concentration) exhibit marked discontinuities interspecifically (Figs. 2 and 3, respectively). Yet the three largest species (*R. catesbeiana, R. pipiens* and *R. clamitans*) indicate no discontinuities with respect to metabolic rate as a function of body weight (Fig. 1).

The observed discontinuities may be integrated into a single generalization if the following assumptions are made: (1) Assume that the *in vivo* respiratory rate of skeletal muscle is conditioned by the cell surface of the muscle. (2) Assume that in the absence of neural stimulation the muscle respiratory rate is low (inhibited). (3) Assume that the surface-dependent respiratory system is activated in an all-or-none fashion by the central nervous system.

If the neurally activated state of the muscle respires considerably faster than the inhibited (non-activated) state, the best approximation of the respiratory rate under neural influence might be given by the product of (a) the frequency of neural stimulation and (b) the surface-dependent respiratory system. If buccopharyngeal frequency can be taken to indicate the general level of central nervous activity, and if one assumes a respiratory system dependent on muscle surface, then one can substitute buccopharyngeal frequency and muscle surface concentration for *a* and *b*, respectively.

The product of (a) buccopharyngeal frequency and (b) gastrocnemius surface concentration has been plotted vs. metabolic rate for the four Rana species in Figure 5. Since:

Buccopharyngeal frequency $= k\,W^{\circ}$ (k a species constant) and: cm.^{2}/cm.^{3} fibers $= k'W^{-\frac{1}{3}}$ (k' a species constant) the product is then:

(Buccoph. freq.) × (cm.^{2}/cm.^{3} fibers) $= k \cdot k'\ W^{-\frac{1}{3}}$.

Both the products and metabolic rates of the intact animals vary directly as $W^{-\frac{1}{3}}$, and are thus directly proportional to each other.

Figure 5 illustrates that inter-specifically the derived numerical quantities (products) approach direct proportionality with respect to resting metabolism of the intact frogs. Given an interacting system such as that suggested, certain characteristics might be anticipated for the elements considered separately.

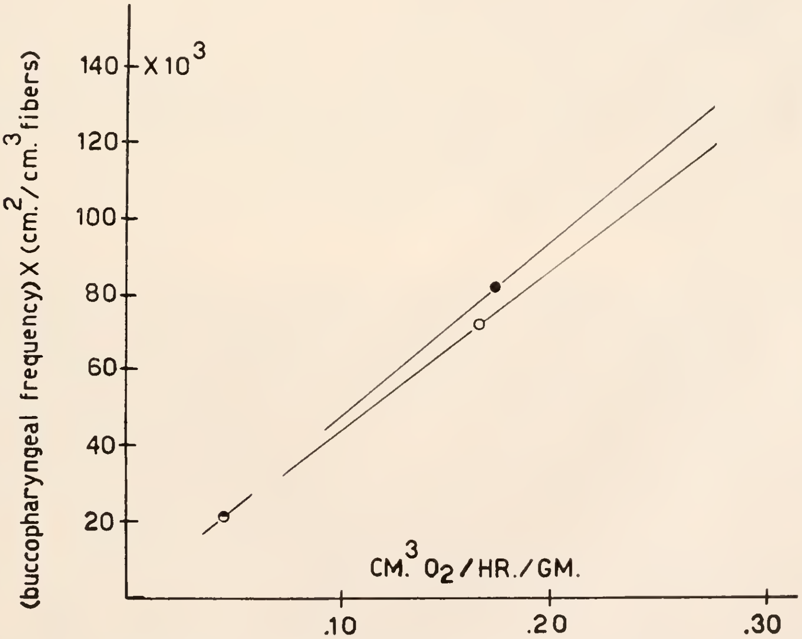

FIGURE 5. A hypothetical, neurally activated, surface-dependent muscle respiration rate plotted vs. resting metabolic rate of the intact frog. Surface concentration values have been multiplied times buccopharyngeal frequencies in the corresponding species (ordinate). The products are plotted vs. resting metabolism in the corresponding Rana species (abscissa). Symbols are as in Figures 3 and 4.

Temperature and respiratory rate

Consider a hypothetical organ which exhibits a positive temperature coefficient for respiratory rate, and which is subjected to periodic all-or-none stimulation from a source also exhibiting a positive temperature coefficient for frequency of stimulation. Assume that the temperature coefficient of the stimulated respiratory rate of the organ is equal to or greater than that of the non-stimulated respiratory rate. Under these conditions, the temperature coefficient of the organ under the influence of the stimulator will be higher than the temperature coefficients of either the non-stimulated respiratory rate or the stimulator.

Thus, one might expect the temperature coefficients of excised (non-stimulated) muscle respiratory rate and of buccopharyngeal frequency each to be lower than the temperature coefficient of intact frog respiratory rate.

Krogh (1914) found a Q_{10} of about 3 for respiratory rate in the intact frog. Morales' study of organ respiration and temperature in the frog would indicate a very low Q_{10} (1.4) for the QO_2 of excised external oblique muscles (Morales, 1943). Kidney and liver respiratory rates also demonstrated Q_{10} values lower than that for whole animal respiration.

Preliminary experiments in this laboratory indicate that buccopharyngeal frequency in resting *R. pipiens* increases from about 45 oscillations/minute at 5° C. to about 120/minute at 25° C., an increase of much smaller relative magnitude than that of O_2 consumption in the intact frog.

The inhibited nature of isolated frog muscle is indicated by the findings of Stannard (1939). He found uptake values of ca. 0.03 cc./hr./gm. (fresh weight) for resting leg muscles at 22° C. The same muscles under direct stimulation (electrical and chemical) exhibited uptake values some 5 times higher. He produced evidence that only the activity increment of QO_2 was mediated via the Warburg-Keilin (cytochrome-cytochrome oxidase) system.

Tissue respiration

Unfortunately, information is lacking concerning the QO_2 of isolated frog muscle as a function of body weight. Information is available concerning muscle QO_2 and body weight in rats (Bertalanffy and Estwick, 1953). The authors found only a slight size dependency of QO_2 with teased musculus femoris from rats between 10 and 380 gms. body weight. Clearly their findings fail to indicate a surface-dependent muscle respiration. However, it should also be clear that muscle as studied by Morales and Stannard is not comparable to the muscle *in vivo*. There also seems no very good reason to presume that findings from mammalian tissue studies are immediately applicable to poikilothermic vertebrates.

The literature prior to 1947 concerned with body size and tissue metabolism has been adequately reviewed by Kleiber (1947). Since that time an extensive analysis of tissue QO_2 and body size has been published by Krebs (1950). While the ratio of rate of heat production is ca. 9:1 for the mouse to horse, the QO_2 of homologous tissues indicate ratios ranging from ca. 2:1 for brain cortex to 5:1 for liver. Krebs concluded that the metabolism of the organs as studied by him would not be adjusted to the thermal requirements of the whole animal.

The preceding observations have one element in common. In all cases intact animal respiratory performance is more than the simple summation of individual tissue respiratory rates. Thus, the temperature coefficients for QO_2 of isolated tissues are lower than the temperature coefficient for the QO_2 of the same tissues considered collectively as the whole animal. Similarly, with respect to metabolism as a function of body weight and species in the mammals, the individual tissues exhibit QO_2 differences of smaller relative magnitude than the differences in the metabolic performances of the intact animals. The present paper has attempted to consider one possible influence on metabolic rate which requires the intact structure for its expression, the central nervous system. The brain has been implicated in a directive role in metabolism during temperature acclimatization by Freeman (1950). Differences in brain metabolism have been correlated with activity in marine teleosts (Vernberg and Gray, 1953), and similar correlations have been reported in studies on the brain metabolism of arctic and temperate fish (Peiss and Field, 1950).

Metabolic rate and cell surface during ontogeny

The correlations which have been made lead to the logical question of the relationship between cell surface and metabolic rate during the whole ontogeny of the animal. Such an inquiry was further prompted by the observation of the dorsal myotome of the tail in mature (5 gm.) *R. pipiens* tadpoles. Camera lucida analysis of these muscles indicated surface concentrations similar to those of adult *R. pipiens* gastrocnemii (ca. 350 $cm.^2/cm.^3$ fibers in 30–40 gm. animals). The musculature of the tadpole tail is resorbed at metamorphosis and the materials reformed in part into the muscles of the resultant frog. The newly formed frog muscle has a much higher surface concentration (ca. 900 $cm.^2/cm.^3$ fibers in 3 gm. *R. pipiens*). Thus, metamorphosis is associated with a marked increase in the surface concentration of the skeletal muscle elements.

Wills (1936) has measured metabolic rate from fertilization to metamorphosis in *R. pipiens*. With my comments inserted parenthetically, his findings may be summarized as follows:

(1) During cleavage and differentiation of the tadpole from the egg, metabolic rate increases in a roughly exponential fashion (surface concentration increases during cleavage and as additional cells are formed from yolk reserves).

(2) During the growth period of the free-living larva, metabolic rate decreases to about ⅓ of its maximum rate (surface concentration decreases due to growth in part by cell enlargement).

(3) At metamorphosis metabolic rate increases as the tadpole differentiates into the frog (metamorphosis is associated with the formation of new skeletal muscle elements with higher surface concentration).

(4) From the present study, metabolic rate decreases during the growth period of the frog proper (post-metamorphosis growth is associated with cell enlargement of the skeletal muscle; hence a decrease in surface concentration).

Thus, the general aspects of metabolic rate changes and changes in cell surface concentration are coincident during ontogeny. The low metabolic rate observed in the fertilized egg (ca. 0.02 cc./hr./gm. calculated from the tabular data of Wills) is comparable with the low surface concentration of the egg (ca. 33 $cm.^2/cm.^3$ for a spherical egg of 1.75 mm. diameter).

Certainly no simple relationship exists between number of cells (and hence surface concentration) and metabolic rate during early development (Tyler, 1942). Developing systems are, by definition, not in a state of simple maintenance, and the energetic requirements of the differentiation process may be quite independent of those of maintenance.

Kleiber *et al.* (1943) have studied metabolic rate during the developmental period of the rat fetus. Philips (1941) has measured QO_2 during the developmental period of the chick embryo. These authors agree that the metabolic rate (metabolism/gm./time) remains reasonably constant over time periods during which the embryo or fetus undergoes large increases in mass. As pointed out earlier in this paper, the formation of new skeletal muscle elements decreases or ceases at about the time of birth in mammals. It becomes a logical necessity to presume that increases in mass prior to birth are, in part at least, the result of cell proliferation. As was indicated previously, in an organism increasing by the addition of cells of fixed size, one might predict metabolic rate constancy. The findings of Kleiber and Philips are consistent with the prediction.

Although cell surface and metabolic rate have been correlated, the question of causality between these quantities remains unanswered. The present study has admittedly regarded the organism as a bundle of muscle fibers, and to this extent it suffers from oversimplification. Being hesitant to regard "organismic phenomena" as unique in origin, I have simply attempted to reconstruct the organism from a consideration of its constituent elements, the cells.

SUMMARY

1. A consideration of cell surface was proposed as an approach to the general problem of body size and metabolic rate in the animal kingdom.

2. Experimental techniques were described concerned with the quantitative measurement of standard metabolic rate and muscle cell surface in frogs ranging from 1 to 350 grams adult weight.

3. It was found that both metabolic rate and muscle surface concentration vary directly as $W^{-\frac{1}{3}}$ for each species considered separately.

4. In the genus Rana, it was assumed that buccopharyngeal frequencies represented general levels of central nervous activity. By further assuming a respiratory system dependent on muscle cell surface, a simultaneous treatment of neural activity and muscle surface was suggested as a possible mechanism of control of *in vivo* respiration.

5. The proposed mechanism was considered in reference to temperature, neural activity, and the respiratory performance of excised tissues and the intact animal.

6. Cell surface was considered as it might relate to metabolic performance throughout ontogeny.

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ACTIVITY IN A CRUSTACEAN GANGLION. II. PATTERN AND INTERACTION IN BURST FORMATION[1]

DONALD M. MAYNARD

Biological Laboratories, Harvard University, Cambridge, Mass.; Marine Biological Laboratory, Woods Hole, Mass.; University of California, Los Angeles, Calif.

Repetitive, coordinated activity is one of the most characteristic properties of neuron aggregations. Because of the anatomical complexity of most of these aggregations, it has also been one of the most difficult to describe in terms of interaction between component units.

Recently in the vertebrate central nervous system (Verzeano and Calma, 1954; Burns, 1951, 1954; Amassian, 1953; Amassian and De Vito, 1954; Li and Jasper, 1953) and earlier in the classical *Limulus* cardiac ganglion (Rijlant, 1932a, 1932b; Prosser, 1943; see, also, Krijgsman, 1952) single unit analysis proved possible only after removal of large portions of the nervous structure or when recording from a small percentage of the active fibers. These studies have consequently led to uncertain interpretations of the mechanism underlying burst activity, for only a portion of the total activity could be followed, and the details of interaction between units could not be determined.

In contrast to the above preparations, the small number of neurons (nine, Alexandrowicz, 1932) in the cardiac ganglion of decapod Crustacea permits single unit analysis of normal activity in the whole ganglion. I shall describe electrical recordings from the cardiac ganglion of *Homarus americanus,* the eastern lobster, and *Panulirus interruptus,* the spiny lobster, and shall present an analysis of the burst in terms of single unit activity. Though involving few units, neither the anatomical nor the physiological organization of the system is simple, and rather complex patterning and integration occur. Preliminary reports have appeared (Welsh and Maynard, 1951; Maynard, 1953b).

Anatomy and Terminology

The decapod cardiac ganglion is one of the most completely described neuronal systems. This is partly because only nine neurons are involved and partly because these form an autonomous system with limited and well defined internal and external connections.

A review of the anatomy may best be given diagrammatically. Figure 1 is derived from the observations of Alexandrowicz (1932) on *Homarus vulgarus* and *Palinurus vulgarus* and from my own observations on *Homarus americanus, Panulirus argus,* and *Panulirus interruptus.* The five large neurons send collaterals to neuropile networks near their somata in the anterior portion of the ganglion. The

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four small units send collaterals to these anterior neuropiles and to additional neuropiles in the posterior part of the ganglion. It is not certain whether the large units connect with these posterior neuropiles. Both large and small units have dendritic arborizations ramifying in the muscle beside the ganglion trunk. Branches of the large cell axons extend throughout the myocardium. The final destination of the small cell axons remains unclear.

Three pairs of nerve fibers from cell bodies in the ventral ganglion chain enter the cardiac ganglion in the paired dorsal nerve. These form the extrinsic system of fibers in the ganglion and are involved in central regulation of ganglionic activity (Maynard, 1953a, 1954). Their function and characteristics will not be considered here.

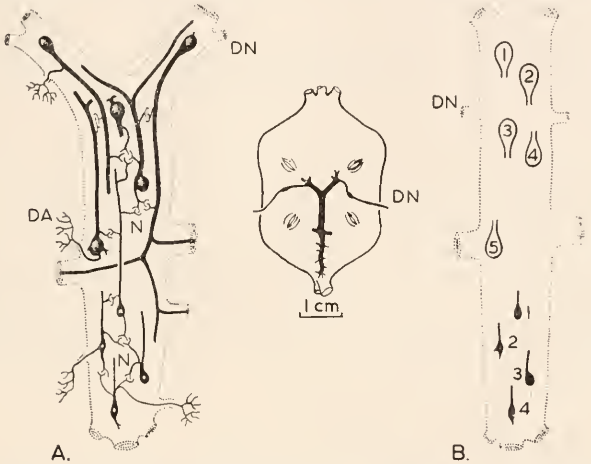

FIGURE. 1. Cardiac ganglion (diagrammatic). A. *Homarus:* relations of cell bodies, neuropiles (N), and dendritic arborizations (DA). Full course of axons is not shown. Extrinsic inhibitor and accelerator fibers entering the ganglion in the dorsal nerve (DN) have been omitted. B. *Panulirus:* positions of cell bodies and direction of major axon. Inset: *Homarus* ganglion trunk *in situ* in heart. Length: width ratio much reduced in all diagrams.

In describing the records, the following terms will be used:

Activity of a single unit:

Run—Repetitive firing at a uniform frequency, continuing for long periods (few minutes to hours).

Train—Repetitive firing for a limited period (usually not more than one second), followed by a period of inactivity. Generally the train is repeated at regular intervals.

Activity in several units:

Volley—Two or more different spikes occurring close together and followed by a period of inactivity: each spike occurs only *once* per volley. The volley must be repetitive and not merely chance superposition of several runs or trains of different frequencies.

Burst—Two or more different spikes occurring close together and followed by a period of inactivity. At least one spike must repeat during the burst, which is therefore generally composed of two or more superposed trains.

METHODS

The first and most complete experiments were performed on *Homarus americanus* (Milne Edwards). Later work on *Panulirus interruptus* (Randall) confirmed and slightly extended the earlier results.

The cardiac ganglion lies on the inner dorsal side of the heart. In early experiments this was approached by a longitudinal cut through the ventral heart wall. The ventral surface of the heart had previously been exposed by removing the head, viscera, and ventral portion of the thorax. In later experiments the heart was first exposed dorsally by removing the overlying hypodermis and carapace. The myocardium above the ganglion was then torn away with forceps to expose the central trunk of the cardiac ganglion. Neither the destruction of the dorsal wall of the heart, the ventral cut, nor the severing of nerves running from the central ganglion trunk into the myocardium had great effect on the heart beat. Only cuts or damage to the central trunk itself caused appreciable change in the burst pattern. In a few instances the heart was removed from the carapace before exposing the ganglion. The preparations in such cases, however, were less likely to be satisfactory.

Ordinarily after exposure silver wire hook electrodes were placed under the ganglion trunk, and side branches connecting it to the muscle were severed so that the trunk could be lifted free of the muscle for recording. Such a preparation was termed a *semi-isolated* ganglion. Anteriorly and posteriorly it retained major motor connections with the myocardium. Complete isolation, in which the ganglion was removed entirely from the heart muscle, usually involved destruction or removal of a portion of the ganglion trunk and certain of the ganglion neurons.

Cole's lobster fluid (1941) or sea water was used as perfusion fluid. Room temperatures ranged between 20 and 25° C. Grass P-3 and P-4 amplifiers were used with single or double beamed oscilloscopes.

A number of ganglia were stained with methylene blue and fixed in ammonium molybdate. These were usually dehydrated, cleared, and mounted for later examination.

RESULTS

The normal nervous activity recorded from the central trunk of the cardiac ganglion forms intermittent, patterned bursts separated by regular silent periods (Rijlant, 1932d). If the extrinsic nerves are intact, impulses arising in the central nervous system and unrelated to the burst pattern may also be recorded (Fig. 2a). In most cases, however, the extrinsic nerves were cut so that only activity originating in the ganglion neurons was present.

Most, if not all, of the nine cells of the ganglion were active in the burst, and most fired more than once (Fig. 2). After the first, typically small impulse, both large and small amplitude spikes appeared. The large spikes then decreased in frequency and dropped out, two or three small spikes remaining active to end the burst. The pattern and order of impulses was remarkably constant. The first few spikes often retained the same order for over 3500 bursts (one hour) even during marked changes in total burst duration. Although *Panulirus* and *Homarus* showed essentially the same pattern of ganglion activity, *Panulirus* bursts frequently tended to last somewhat longer because of prolonged small spike activity.

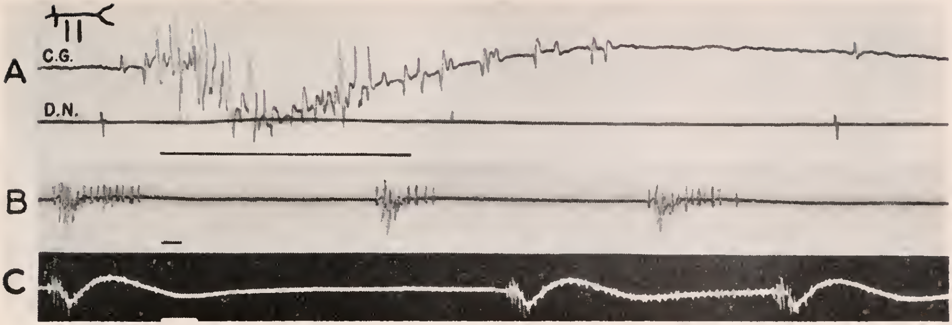

FIGURE 2. A. Normal burst: simultaneous records from the ganglion trunk (C. G.) and the dorsal nerve (D.N.). The two impulses in the dorsal nerve originated in the ventral, thoracic ganglia. Diagram indicates position of recording electrodes on ganglion trunk. B. Alternating burst. C. Double burst following a normal burst. All records from semi-isolated *Homarus* preparations. Time scale, 0.1 second.

Occasionally an *alternating rhythm* (Fig. 2b) or *double bursts* (Fig. 2c) occurred (see Rijlant, 1936). In the alternating rhythm the longer discharge was followed by a slightly longer silent period. The spike pattern was consistent within either short or long bursts, but differed between the two. In the double burst the terminal small spike activity continued two or three times longer than usual until large spikes again appeared. The pattern of this second half of the burst was not like that of the first, and there were fewer large spikes.

TABLE I

Gross parameters of burst activity (Homarus, ten animals)

Parameter	Mean value	Range
Heart (burst) rate	0.84 per second	0.35 to 1.30
Burst duration	0.32 second	0.11 to 0.55
Spikes per burst	54	19 to 96
$\frac{\text{Interburst duration}}{\text{Burst duration}}$ (ratio)	2.9	0.8 to 8.8

Gross parameters of burst activity were measured in ten *Homarus* immediately after preparation (Table I); these were semi-isolated ganglia. The heart (burst) rate is somewhat lower than that measured *in vivo* by Burger and Smythe (1953), which ranged between 0.87 and 2.27 beats per second.

Muscle potentials

The slow potential changes recorded in semi-isolated ganglia (Figs. 2 and 4) are probably muscle action potentials. They are comparable in shape and duration to potentials recorded from the cardiac muscle by gross electrodes and by intracellular glass capillaries and are absent in isolated ganglia. Slow potentials occurring before and during the burst, as observed in *Limulus* (Prosser, 1943; Rijlant, 1932a; Heinbecker, 1936) were not obvious. The small changes found in isolated ganglia usually resembled summating after-potentials (Fig. 9).

Burst analysis

"Normal." The activity of a typical preparation is more fully analyzed in Figure 3. Eight spike types were identified in the burst. The large spikes had the greatest conduction velocity and traveled posteriorly, while the smaller, slower impulses ran from the posterior ganglion forward. The origins of these impulses corresponded with the anatomical positions of the large and small neuron somata, respectively (Fig. 3, diagram, and Fig. 1). This association of large spikes with the large cells and the small spikes with the small cells has also been supported by the results of mid-ganglion transection, which abolished most small spikes in the anterior portion containing large cell bodies and all large spikes in the posterior portion containing small cells.

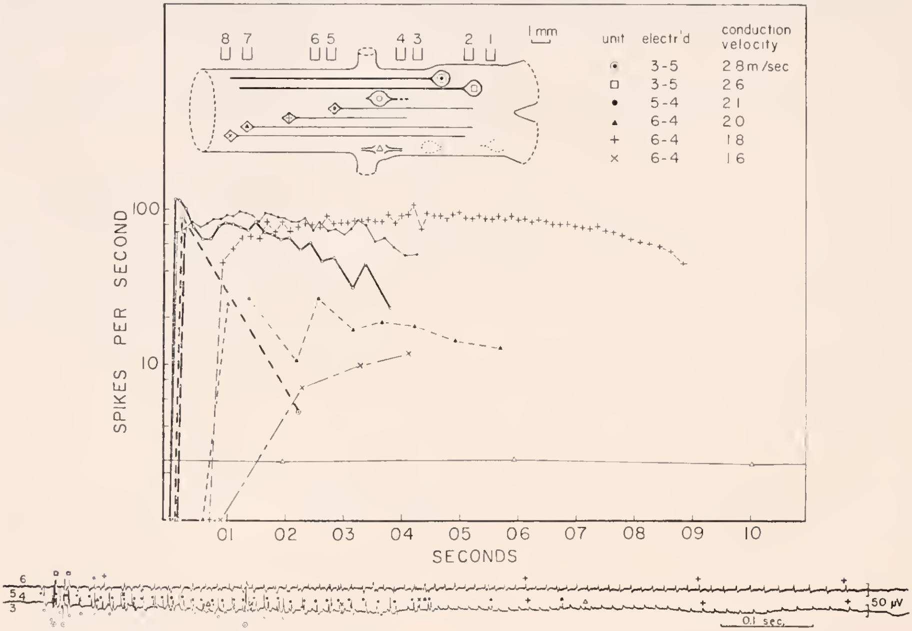

FIGURE 3. Burst analysis: multiple electrode recording. Bottom, recording from electrodes 3–4 and 5–6. Graph, analysis of burst into discharge frequency of component units as recorded from electrodes 3–4. ○, ⊙, and □ represent presumed large cell activity. Diagram of ganglion trunk indicates position of eight recording electrodes (anterior to right). Schematic somata represent origin of impulses; schematic axons, the extent each impulse was conducted. Maximum standard deviation of conduction velocity values, about 15%. *Electr'd,* electrode numbers between which velocities were measured. Temperature, 25° C. *Panulirus,* semi-isolated.

Two of the impulses recorded from the central ganglionic trunk were not conducted. They remained constant in duration and amplitude; one was involved in the burst pattern and one was not. They possibly represent either local, subthreshold activity or blocked impulses and may arise in neuropile collaterals or in main fibers and cell bodies.

Neither in this preparation nor in others has it been possible to determine the neuron producing the first impulse of the normal burst. The small amplitude suggests the first small cell, but the fifth large cell cannot be completely excluded.

A curious but consistent aspect of the train pattern of many units was the oscillating frequency shown especially clearly in unit ○, Figure 3 and units 1 and 2, Figure 6.

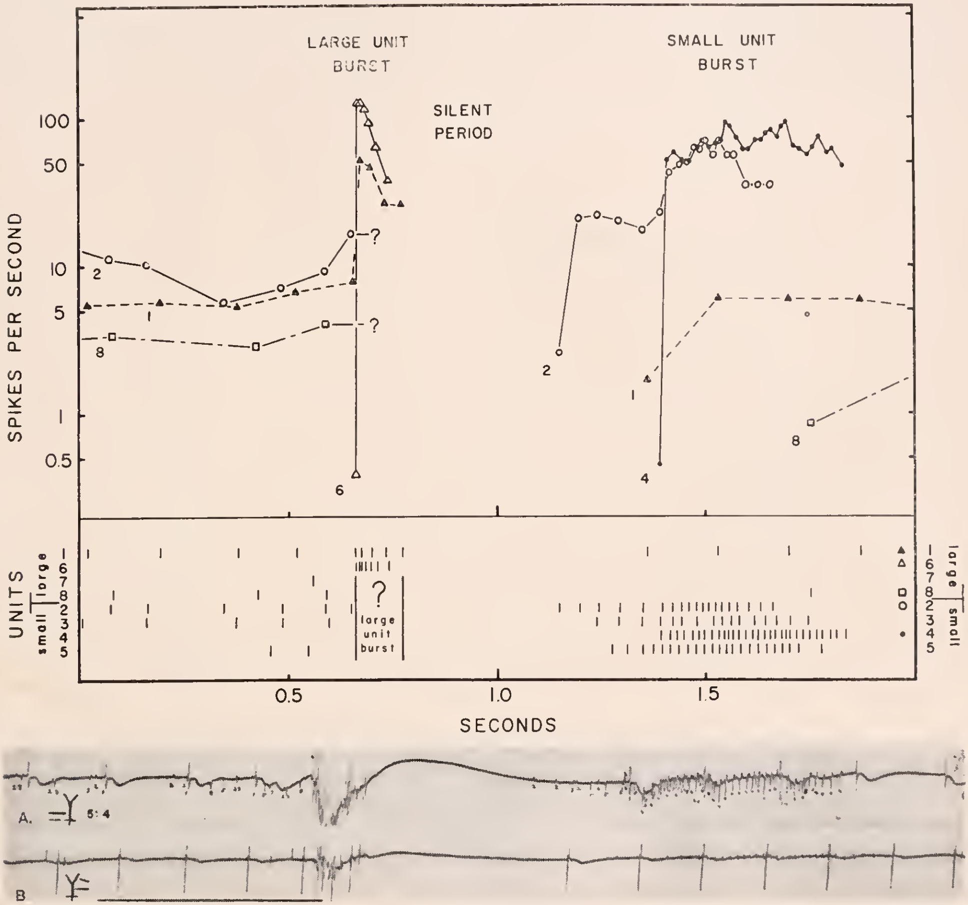

FIGURE 4. Two independent bursts within single ganglion. Records: B follows A by some minutes and after replacing electrodes, see diagrams; 5:4 beside diagram indicates number of large and small units present in preparation. Time scale, 0.5 seconds. *Homarus*, semi-isolated. Graph: plot of unit frequencies from record A. Units 3, 5, and 7 omitted for simplicity.

"Abnormal." In a few, apparently damaged preparations, two independent bursts were present (Fig. 4, see also, Heinbecker, 1933 and Rijlant, 1936). One burst included both large and small units, while the other included small cells only, and originated in the posterior part of the ganglion, suggesting two morphologically separate regions of interaction. Several other points are of interest in this prepa-

ration: 1) The large unit burst and each isolated large cell impulse were followed, after a latency of 10–14 msec., by a muscle action potential. The small cell burst was not. 2) Several units, both large and small, tended to fire in independent runs as well as in the burst. 3) A compensating silent period in the activity of a running unit followed the high frequency discharge during the burst (unit 1) so that its mean frequency was unchanged.

Isolated portions of the ganglion frequently retained burst activity. In one segment containing only three large cells (numbers 3, 4, and 5, Fig. 5) seven spike types were identified. Since there was no close correlation between any of the patterns of activity, each spike type probably represented a separate neuron, some of which must have lacked cell bodies. Their small amplitude and monophasic nature suggest that most of the impulses were local, rather than conducted, potentials. This, if correct, implies that neither cell bodies nor conducted impulses are necessary for synaptic (?) interaction. The neuron soma, however, may be essential for maintained, spontaneous activity (see below).

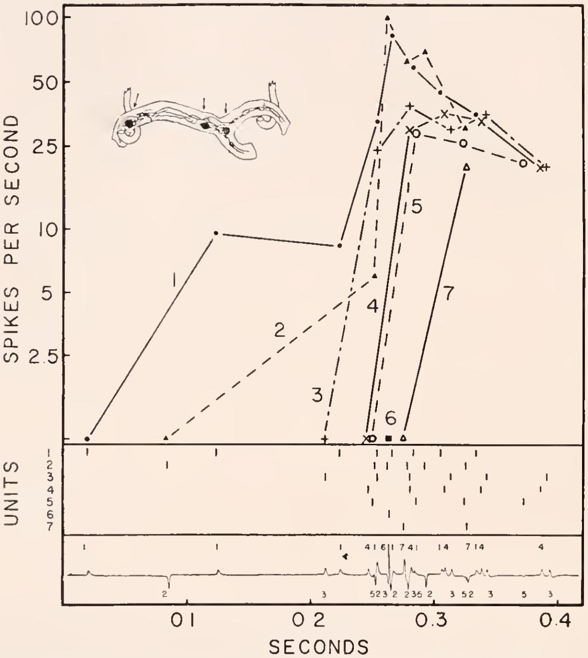

Figure 5. Burst analysis from portion of ganglion containing large cells 3, 4, and 5 only. Inset shows recording electrodes and large cell bodies (arrows). *Homarus*, isolated.

Run activity

In a number of preparations (Figs. 3, 4, 6, and 8) large and occasionally small cells fired in continuous independent runs rather than intermittently in synchronized, high frequency trains. Such runs could continue for hours at relatively constant frequencies, usually between 2 and 15 impulses per second. They seemed to result from procedures which isolated units from external phasic stimulation, for they could be produced by direct inhibition via the extrinsic nerves of all but one or two large cells, as well as by a surgical isolation of one to three cells, damage to synaptic or neuropile areas, or application of high concentrations of acetylcholine (see Prosser, 1943, for similar effects of ions). Further, unit activity could shift from runs to trains and back without changing the mean frequency of discharge.

The decay from intermittent train to continuous run activity was occasionally observed to be directly associated with a progressive decrease in synaptic influences (Fig. 6). As both the mean activity and activity per burst of the driving unit (unit 1) decreased and the mean activity of the driven unit (unit 2) did not, unit 2 fired fewer times and reached a lower maximum frequency per burst. At the same time, it began firing before unit 1 in the burst. Finally the distinction between activity and silent period disappeared, and an oscillating run resulted. Units 3, 4, and 5 responded with increased activity only in the largest of the bursts and possibly required both temporal and spatial summation of activity in units 1 and 2. It

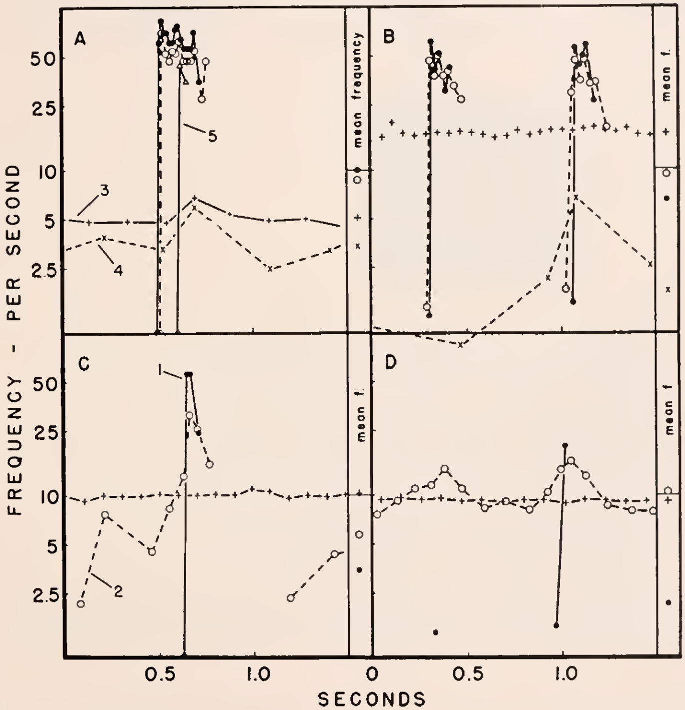

FIGURE 6. Transition from train to run patterns. A, B, C, and D, analyses of successive records from a single ganglion at several-minute intervals. Mean impulse frequencies were determined over a period long in respect to duration of burst and silent period. Note resemblance of pattern in C to those in Figures 4, 5, and 7. See text. *Homarus,* isolated: 2–3 large cells, 1–2 small cells.

should be noted that the first unit to fire in a burst is not necessarily the most important in producing or maintaining the burst.

Apparent isolation did not invariably produce runs, for occasionally, especially in small units, the train pattern continued with no evidence of activity in other neurons. Neither runs or trains, however, were observed in portions of the ganglion trunk lacking cell bodies. Nor, where cell bodies were present, did spontaneously active units exceed the number of neuron somata.

Interaction

Most ganglion interaction was of a "diffuse" nature, characterized by long latencies, repetitive responses in the post unit, and extensive spatial and temporal summation. Occasionally synchronization between spontaneously active units produced volleys rather than bursts. In such cases, very long and variable latencies were present (17 to 48 msec.).

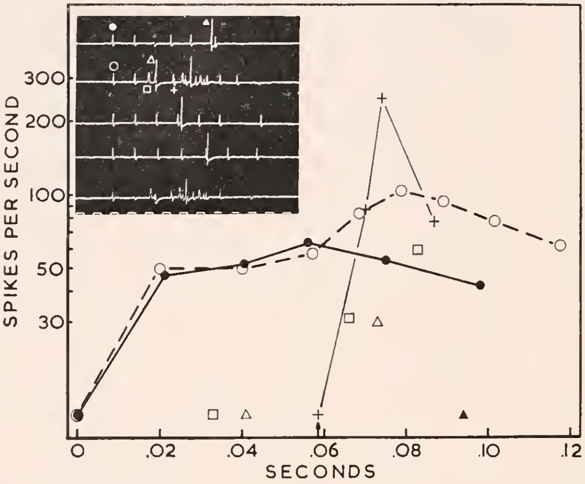

FIGURE 7. Reciprocal interaction. Inset, records of five successive bursts; graph, superposed analyses of first two bursts. Filled symbols, first burst; open symbols, second burst. Arrow, first impulse of unit 4 (+) in second burst. Time cycle on records, 60 per second. *Homarus,* isolated.

Reciprocal or reverberating interaction between the neurons was probably normally present. In an unusually clear example of this (Fig. 7) every third burst contained four spike types while the first and second burst of the cycle contained only two. A longer silent period followed the larger bursts. The coordination between unit 1 (○) and the complex of units 2 (△), 3 (□), and 4 (+) suggests that activity in unit 1 initiated activity in the other units. Reciprocally unit 4 apparently stimulated unit 1 to increased activity. The resemblance between the burst pattern of this preparation and those of Figures 4, 5, and 6 should be noted.

Run activity in an individual unit shifted to synchronized train activity during a burst in two ways:

1. Units active in runs changed to train activity in conjunction with the reappearance of inactive units (Figs. 4 and 8). Frequently these latter formed a "burst complex" of several spikes which acted in an all-or-none fashion as a single "unit." The cells previously active in runs were simply added to this burst. Their increased frequency during the burst, however, was compensated by a following silent period.

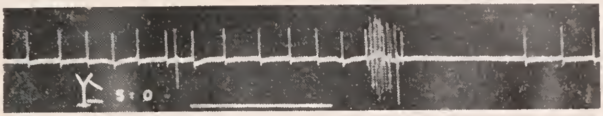

FIGURE 8. Incorporation of run pattern into "burst complex." Time scale, 0.5 second. *Homarus*, semi-isolated; small cells lacking.

2. The burst appeared by the gradual change from largely asynchronous activity in several units to brief synchronized trains. In the instance figured (Fig. 9A, B, C), this synchronization coincided with the establishment of more similar mean frequencies in the separate units. The simple burst reactivated a "burst complex" of the type mentioned above, and drove it at a somewhat lower frequency (Fig. 9D). In this case the spikes of the simple burst probably arose in the three neurons with somata, while those of the burst complex, as in Figure 5, probably arose in fibers lacking somata.

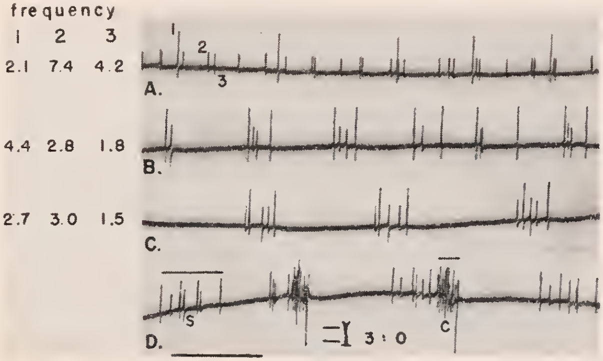

FIGURE 9. Condensation of run patterns into simple burst. A, B, and C, at several minute intervals after isolation. Mean spike frequencies of three active units to left of records. D. Several minutes later, "burst complex" (C), involving additional impulses is superposed on and driven by simple burst (S). Time scale, 0.5 second. *Homarus*, isolated: three large somata only.

Function of units

Large neurons. The large cells are considered the major motor neurons: 1) In bursts (normal, double, and separate, see Figs. 2 and 4) and with independently firing units, the muscle twitch and action potential are associated with large unit activity only. 2) The small cells may be removed without destroying the heart beat in the portions which remain innervated by the large cells.

Since, however, the large units probably influence activity in the small cells (Fig. 4) and since they maintain the burst without small neuron somata, they fulfill interneuron and pacemaker functions. Further, if their dendritic arborizations are sensitive to stretch (Alexandrowicz, 1932; Bullock, Cohen and Maynard, 1954) the large units also serve as receptors.

Small neurons. The premature large cell activity of the double burst is lost when the small cells, and consequently their long terminal activity, are surgically removed. In *Panulirus,* the frequency of the large unit bursts is greater when small units remain active during the interburst period. And when excitability of the ganglion is lowered during extrinsic inhibition (Maynard, 1954), the large cells are obviously driven by the small unit activity. Therefore, because (exceptions, unit 1, Fig. 4) small cell activity usually increases activity in the large units, and because they may fire in independent bursts, the small neurons must be considered as interneurons and as pacemakers. Because of their dendritic arborizations, the small cells may also be receptors.

TABLE II

Characteristics of large and small neuron activity

Characteristic	Large neurons	Small neurons
Impulse amplitude	large	small
Conduction velocity:		
Homarus, one animal 25° C.	2.0–3.0 m/sec.	1.4 m/sec.
Panulirus, five animals 23–26° C.	2.4±0.5 m/sec.	1.3±0.4 m/sec.
Motor response	twitch	none ?
Pattern in burst:		
Duration	short	long
Number of spikes	few	many
Maximum frequency per unit	high	low
Tendency to spontaneous runs	great	smaller (not certain)

The function of branches of the small cell axons entering the cardiac muscle is unknown. They may induce a slow contraction, as has been found in other crustacean muscles (van Harreveld and Wiersma, 1937; Kuffler, 1954), which is normally masked by the much greater twitch response to large cell activity. Or, as a second possibility, they may innervate the ostia, for Needham (1954) finds in an isopod that ostial and heart contraction may be separated.

Since a single ganglion neuron may combine several functions, it is impossible to speak of pure pacemaker neurons, receptors, interneurons, or even motor neurons. One may say only that the large cells cause a muscle twitch, the small do not.

The differences between large and small unit activity are summarized in Table II. Conduction velocities were determined with two or more pairs of recording electrodes (see Fig. 3).

Acetylcholine effects

Earlier work has shown that the crustacean heart is quite sensitive to acetylcholine (Welsh, 1939, 1942; Davenport, 1941, 1942). In the present experiments acetylcholine chloride (1 : 1000 by weight) first increased the spontaneous frequency of individual ganglion cells and then caused block. These effects were reversible.

The action on bursts was more complex. If uncoordinated activity and abortive bursts were present, lower concentrations (1 : 100,000) of acetylcholine changed this to regular patterned bursts. At greater concentrations the burst frequency tended to increase, but the compact bursts often began to disintegrate. Units began firing in the interburst period. Some units dropped out, and the interaction between the rest disappeared until each active unit fired in a continuous independent run with its own characteristic frequency. By this time, muscular potentials ceased. The units active at these high concentrations had large spikes and were presumably the large motor cells.

Methylene blue effects

Preliminary observations indicate that methylene blue increases the excitability of the ganglion cells. A concentration of 5 : 1000 caused a marked increase in frequency, especially of the large motor units. Ganglia remained alive and functioning, presumably near normal, several hours after application of the methylene blue and after the cells were stained. The physiological response to the stain was noted before color began to appear in the neurons.

Discussion

Two alternative mechanisms could yield the rhythmic, coordinated burst found in the cardiac ganglion. In the *Multiple Chain* (Fig. 10a) only morphological pacemakers are spontaneously active. Their basic rhythm determines the heart beat frequency and they in turn stimulate second and third order neurons, whose response characteristics determine the burst pattern. Reciprocal interaction is absent. In the *Closed Chain* (Fig. 10b) every neuron is spontaneously active and reciprocal interaction is present. Both the heart rate and the form of the burst are therefore a function of the whole aggregate.

Rijlant (1932a, 1932b) has suggested that a multiple chain mechanism with specific pacemakers operates in the *Limulus* ganglion. The present results from lobsters, however, agree more closely with a closed chain mechanism. The great similarities between records from *Limulus* and the decapod cardiac ganglion suggest that these differences rest on differing interpretations rather than on different phenomena. If this is correct, the many additional units in the *Limulus* ganglion do not add basic parameters to its activity, and both lobster and *Limulus* probably have a common mechanism of burst formation. The activity of the decapod cardiac ganglion is therefore probably not a special case resulting from the small number of its component neurons.

Mechanism of burst formation

First, it seems reasonable to conclude that *every* neuron in the ganglion may be capable of spontaneous activity, for most units showed activity when carefully isolated. It is unlikely that this independent run activity represents injury discharges, for: 1) The mean frequency of a unit was not markedly greater during a run than when firing in trains in bursts (Figs. 4 and 6), and 2) a reversible transition between runs and trains was frequent. Furthermore, 3) some of the ways of producing run activity, *e.g.*, inhibition, could not lead to neuron injury.

Second, interaction seemed to occur between all units in the ganglion so that the driven unit reciprocally stimulated the driving unit or "pacemaker" (Fig. 7). This is presumably responsible for the patterned, high frequency, synchronized discharge obtained during the burst. The duration and pattern of the burst certainly depend upon the characteristics of the whole ganglion rather than upon any single neuron.

The occasional train found in "isolated" units was modified by activity in other neurons. Because of this, and because bursts were frequently composed solely of units showing only run activity when isolated, the intrinsic train pattern cannot be essential in the mechanism of the formation of a coordinated burst.

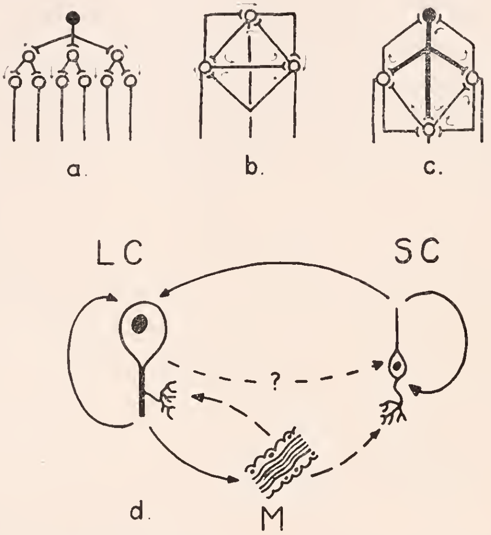

FIGURE 10. "Mechanisms" of burst activity. A. *Multiple chain.* Top cell, pacemaker; second row, interneurons; bottom row, motor neurons. B. *Closed chain.* No morphological pacemaker, every unit acting as pacemaker, motor neuron, and interneuron. Equivalent connections between all units. C. *Modified closed chain.* Non-equivalent connections between units. "Pacemakers" (top cell) in limited sense, but reciprocal interaction and flexibility remain dominant. Probably analogous to organization of cardiac ganglion; see discussion. D. Functional relations within decapod heart; see discussion. LC, large ganglion neurons; SC, small ganglion neurons; M, cardiac muscle; arrows = "——stimulates ——"; dashes indicate uncertainty.

The two parameters, impulse activity during the burst (frequency and duration) and burst frequency are not independent (Fig. 2). The silent period, which corresponds with a period of lowered electrical excitability (Rijlant, 1932c; Maynard, 1953b) and follows a period of excitation, probably represents a *post-excitatory depression.* For a single unit its duration is proportional to the difference between the level of spontaneous activity and the average frequency obtained by the unit during the burst. Thus, the mean frequency of the unit is not appreciably changed by a shift from a spontaneous run to intermittent trains (Fig. 6).

The individual unit behaved as though balanced between a tendency to fire spontaneously and to respond to external stimulation. At one extreme, spontaneity was reduced or absent, perhaps following removal of the soma, and the unit fired only in response to external stimulation. Such responses could, however, be repetitive and form part of a "burst complex." At the other extreme, all presynaptic stimulation was removed or the spontaneity of the unit was raised without a corresponding potentiation of synaptic transmission, as when acetylcholine was applied (Schallek and Wiersma, 1948) and the unit fired continuously in a run. In the normal burst (Fig. 3) probably only the first unit to fire did so spontaneously (see, however, Figs. 4, 5, 6, and 7). Presumably the rest were ordinarily stimulated to fire before recovering sufficiently from the previous burst to fire spontaneously, and were therefore maintained in a "sub-spontaneous" excitability state.

Though reciprocal interaction clearly exists, the "burst complex" (Figs. 5 and 8) and certain "pacemakers" (Figs. 4 and 6) imply that not all units have equal stimulating influence. The pure closed chain, therefore, may be modified to include some units (Fig. 10c) which make more numerous and more effective efferent connections. These probably fire early in the burst, driving other units. The driven units in turn seem to react on the pacemaker, each other, and with additional units until all neurons in the ganglion are activated. Under experimental conditions this "pacemaker" may be removed or its activity delayed so that other neurons become active before it. Presumably if sufficient efferent connections are present (bottom unit, Fig. 10c), these cells become secondary "pacemakers" (unit 4, Fig. 4) by stimulating several other units. If sufficient connections are lacking (side units, Fig. 10c), the active units may interact with each other to produce a loose, "simple burst" (Fig. 9c) or volleys. Such units, however, may reactivate the normal "pacemaker" which then serves as an interneuron driving additional units to produce the "burst complex."

The "reverberating circuit" of this ganglion does not seem to function to maintain spontaneous activity in a self-reexciting chain, but to provide means for spontaneously active units to undergo a slower alternating auto-excitation and depression. This implies that there is no single cell whose basic rhythm is the same as that of the recorded burst but that the rhythm of the burst is a function of the interaction of the units and depends upon the integrity of the entire system. Furthermore, the source of spontaneity must reside in each component neuron, not in the interaction between units. Experiments with the cardiac ganglion illustrate how flexible such a system may be, for though the pattern and frequency of the burst may be changed with the removal of units, a coordinated burst not qualitatively different from that of the whole ganglion may continue when over half the component neuron somata have been removed. Similarly functioning circuits may underlie the spindle bursts found in the vertebrate thalamus (Verzeano and Calma, 1954) or bursts from vertebrate cortical slices (Burns, 1951, 1954), and possibly other low frequency rhythms in neuron aggregations.

Interaction and slow potentials

It has been assumed above that most interaction between the ganglion neurons is synaptic, occurring in the neuropile regions. The presence of "simple" and "complex" bursts, however, suggests that the possibility of further interaction between adjacent neurons (Katz and Schmitt, 1940; Arvanitaki, 1942) cannot be excluded.

The present failure to observe slow potentials probably results from the small number of decapod cardiac neurons and their relatively wide separation, both of which diminish summation effects. In *Limulus* the neurons are closer together and more numerous, and slow potentials are seen (Rijlant, 1932a, 1932b; Heinbecker, 1936; Prosser, 1943). Preliminary intracellular recordings (Bullock and Hagiwara, personal communication) show slow potentials in individual neurons of the lobster ganglion which appear to last for the duration of the burst. At present it is not known whether such slow changes function in synaptic transmission in the ganglion, or whether they indicate a form of post-synaptic response.

Large and small neurons

The differences in activity between the large and small cells of the ganglion correspond with the differences found between large and small afferent fibers in doubly innervated sense organs (Katsuki *et al.*, 1950; Bullock, 1953). In both systems the small fibers are less prone to adaptation, giving tonic discharges rather than the brief phasic bursts of the large fibers. The small fibers also have a lower maximum frequency of discharge. In the sense organs the small fibers are the more sensitive. This is not so clear in the ganglion, but the small units frequently appear before the large units enter in the burst, and during inhibition the small units are the most difficult to inhibit.

There are further resemblances between the ganglion activity and the motor supply to doubly innervated crustacean and insect muscles (van Harreveld and Wiersma, 1937; Wright and Adelman, 1954; Wright and Coleman, 1954; Pringle, 1940). The motor fiber causing a twitch response usually has the larger diameter, a higher threshold to reflex stimulation, and tends to give a brief discharge. The small motor fiber causes a slow contraction in the muscle, and like the small ganglion neurons, usually responds first in reflex stimulation giving an extensive repetitive discharge.

"Behavior"

It is tempting to regard the decapod heart as a model organism which is capable of very simple behavior patterns, *e.g.*, rhythmic, coordinated contractions. Its "brain," the cardiac ganglion, shows more resemblances to central nervous systems than to most peripheral ganglia in that it is relatively autonomous, spontaneously active, capable of integrative and patterned activity, and makes afferent and efferent connections with a "complete" effector system. An analysis of activity in the ganglion therefore becomes a description of motor behavior in terms of events in the integrative nervous system. Though such an analogy between a heart contraction and activity patterns in a whole organism is rather tenuous, it does indicate the type of neural integration underlying a simple but coordinated motor response.

Figure 10d summarizes the general relations between the ganglion neurons and the heart. Extrinsic fibers are omitted. Interaction is most evident between the large and between the small units. The small units apparently stimulate the large, but the reciprocal effect has not been as clearly demonstrated. The large units cause the quick tetanic contraction of the heart muscle. This contraction probably affects the ganglion by stretching the arborizing endings in the muscle and possibly lowering the thresholds of individual neurons to permit increased spontaneous or post-synaptic activity. Attention should be called to the differences between this diagram and those presented previously (Heinbecker, 1933; Krijgsman, 1952) in that the small cells are not considered the primary motor units, and the large cells are not considered exclusively as pacemakers. Perhaps most conspicuous is the large number of functions which any single neuron may perform, *i.e.*, act as "pacemaker," "interneuron," receptor, and motor neuron.

The invaluable aid of Dr. John H. Welsh, at whose suggestion this preparation was used and in whose laboratories the major work was done, should be acknowledged. The hospitality of Dr. S. Kuffler at Woods Hole and the kindness of Dr. T. H. Bullock in reading and criticizing the early manuscript must also be mentioned.

SUMMARY

1. The pattern of nervous activity in a spontaneously active nerve center containing only nine neurons, the cardiac ganglion of the lobster, is described. This pattern is a burst of large and small nerve impulses, each neuron firing several times during the burst, followed by a lengthy silent period.

2. When isolated, single large neurons tend to fire spontaneously at a constant frequency several times greater than normal burst frequency. It is suggested that the rhythmic burst derives from such spontaneity by a reciprocal interaction among the ganglion units which produces alternating periods of high synaptic excitation and post-excitatory depression.

3. Large, rapidly conducted impulses originating in the five large ganglion cells cause the rapid contraction of the heart muscle. Smaller, slower impulses arising in the four small ganglion cells produce no noticeable motor response, but increase activity in the large neurons. Both large and small cells seem to function as pacemakers, interneurons, and possibly as stretch receptors as the occasion demands.

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STUDIES ON MARINE BRYOZOA. VI. ANTARCTIC ESCHAROIDES

MARY D. ROGICK

College of New Rochelle, New Rochelle, N. Y.

The writer wishes to express her most sincere appreciation to the National Science Foundation for research grants which have greatly aided this work, and to the Smithsonian Institution for the loan of the U. S. Navy's 1947–48 Antarctic Expedition collection of Bryozoa (collected by Comdr. David C. Nutt) on which this study is based.

The purpose of the present study is to describe two new species of *Escharoides* from the Antarctic and to add further morphological and distributional data on two other rarely found but very confusing *Escharoides* species.

Family Exochellidae is represented by five species in the U. S. Navy's 1947–48 collection: *Exochella longirostris, Escharoides barica, E. bubeccata, E. praestita,* and *E. tridens*. *Exochella longirostris* Jullien 1888 is not restricted to the Antarctic but the four above *Escharoides* species are all Antarctic, so far as is known at present. *Escharoides praestita* was briefly described by Waters in 1904 and has not been reported since. *Escharoides tridens* was briefly described by Calvet in 1909 and two subsequent reports (by Livingstone, 1928 and Vigeland, 1952) added only distributional data but did not amplify the original description; hence it was possible to add considerable new morphological data on both species from the current U.S.N. specimens.

DATA ON SPECIES

Escharoides barica, n. sp.

Figures 1–7

Name derivation. This species was named for the massive "shoulders" (L. *baricus,* "topheavy") produced by the two large avicularial chambers situated one on each side of the peristome.

Diagnosis. Colony encrusting. Zooecia large. Zooecial frontal areolate, rather flat except for the two "shoulder" avicularial chambers and the semicircular peristome. A few (about 5) small pores outline the avicularial chambers. Strongly hooked avicularia recessed back of the abrupt ends of the peristome. Mandible thimble-shaped, scooped, with scalloped tip. Large terminal orifice rounded, devoid of lyrula, cardelles or spines. Ovicell globose, immersed, nonporous but surrounded by areolae.

Measurements. The first figures are the minimum, the next the maximum and the last, in parentheses, the average of 10 readings (usually) for each structure. Readings are in millimeters. L means length, W width.

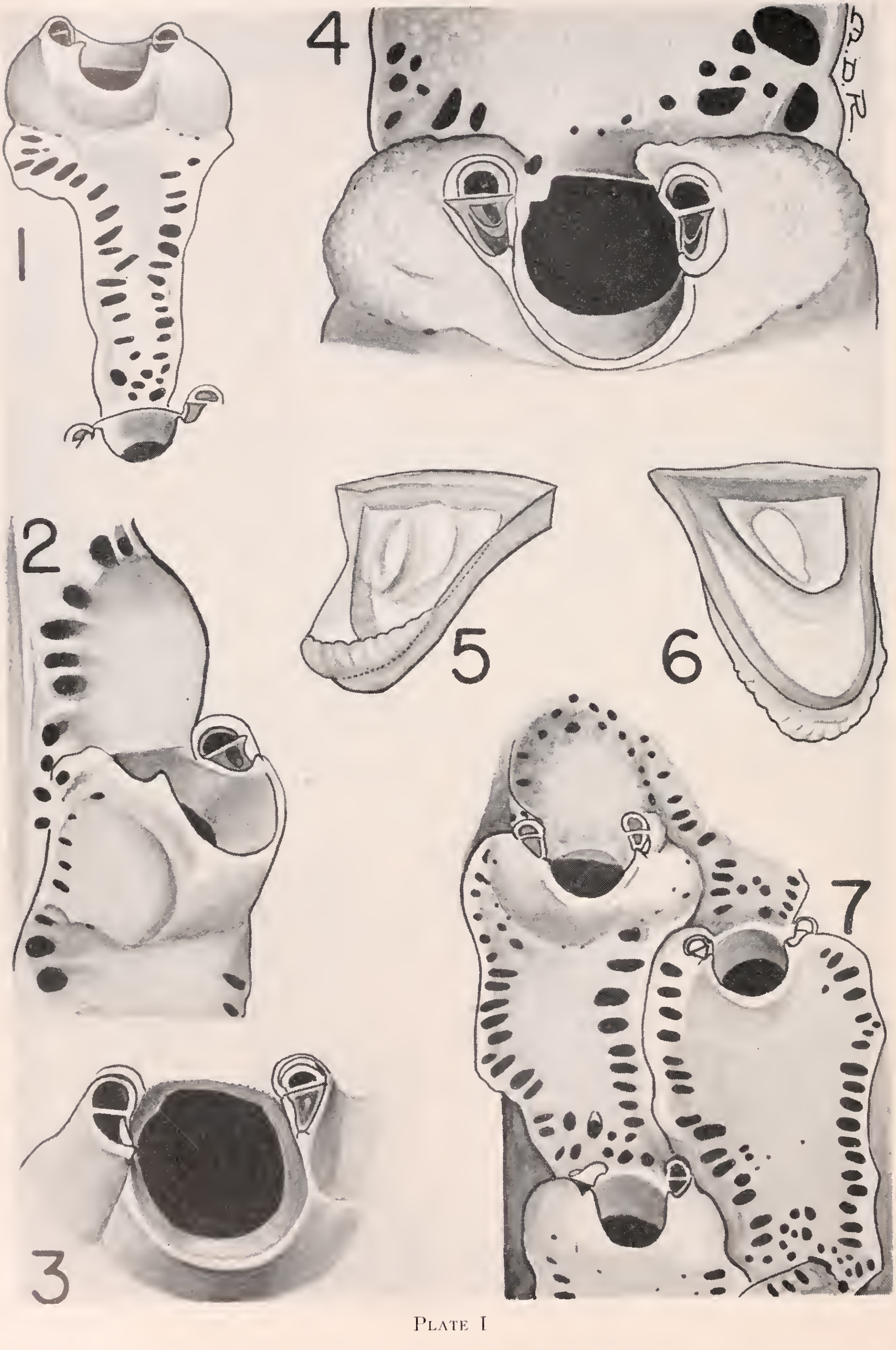
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Plate I

1.469–1.944	(1.738)	L Zooecia
0.936–1.224	(1.037)	W Zooecia
0.216–0.274	(0.239)	L Avicularia
0.130–0.173	(0.154)	W Avicularia
0.317–0.389	(0.349)	L Primary orifice
0.331–0.389	(0.363)	W Primary orifice
0.374–0.518	(0.464)	L Secondary orifice
0.346–0.403	(0.383)	W Secondary orifice
0.101–0.144	(0.124)	L Mandible
0.101–0.130	(0.111)	W Mandible
0.475–0.605	(0.540)	L Ovicell (4 readings)
0.533–0.619	(0.572)	W Ovicell (4 readings)

Zoarium. Only two incomplete colony fragments are in the collection. The bigger one, holotype, from Sta. 44, is 9 mm. by 15 mm. and has about 66 zoids, 4 of which have ovicells. The smaller, younger one, paratype, from Sta. 190, has only 9 orifices (zoids or parts).

Zooecia. Zooecia large, well defined. Edge sometimes a faintly salient thread. Frontal wall an areolated, slightly beaded pleurocyst. Areolae oval, medium-sized, penetrate through innermost layer around the edge. Ribs not strongly developed. The larger part of the frontal is flat to faintly convex. The broader distal part bulges and leans forward slightly from the basal surface. Its bulge is due to the two large avicularial chambers and the projecting peristome. The whole aspect reminds one of well developed shoulders and chest. Back wall very thin, irregularly undulating. Front wall of medium thickness.

Avicularia. Two medium-sized avicularia are present on all fully matured zoids. The avicularia are of uniform size, appearance, location and parallel orientation. They are located on the "shoulders" (avicularial chambers), immediately bordering the orifice, at the very ends of the peristomial semicircle. Their beaks are provided with a sharply hooked tip. Unless the colony is tipped forward so one can see the avicularium and mandible from the top, one is apt to be misled into thinking that the beak is sharply acuminate. However, the top view shows the beak to be very gently triangular (*i.e.,* with tip rounded off instead of acuminate). The mandible is chitin-reinforced around the edge, and a second smaller,

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Explanation of Plate I

All figures on this plate are of *Escharoides barica,* n. sp., and were done with the aid of a camera lucida. Figure 1 is from the Sta. 190 paratype, the remaining figures are from the Sta. 44 holotype.

Figure 1. A zoid showing the large "shoulders" (avicularial chambers) and the two hooked beak avicularia topping them. Zooecial front below them flat and narrowed.

Figure 2. Side view of an ovicelligerous zoid. The left avicularium shows, but the right one, although present, is hidden from this angle. Ovicell partly immersed, bordered by areolar pores.

Figure 3. Primary and secondary orifices and two avicularia on a zoid.

Figure 4. Avicularia and orifices of another zoid.

Figure 5. Inner surface of an open mandible. The scalloped membrane is invisible when the mandible is closed.

Figure 6. The reverse (outer) surface of a mandible showing chitinous reinforcements darkened and the oval thinner "lucida."

Figure 7. One ovicelligerous and two non-ovicelled zoids.

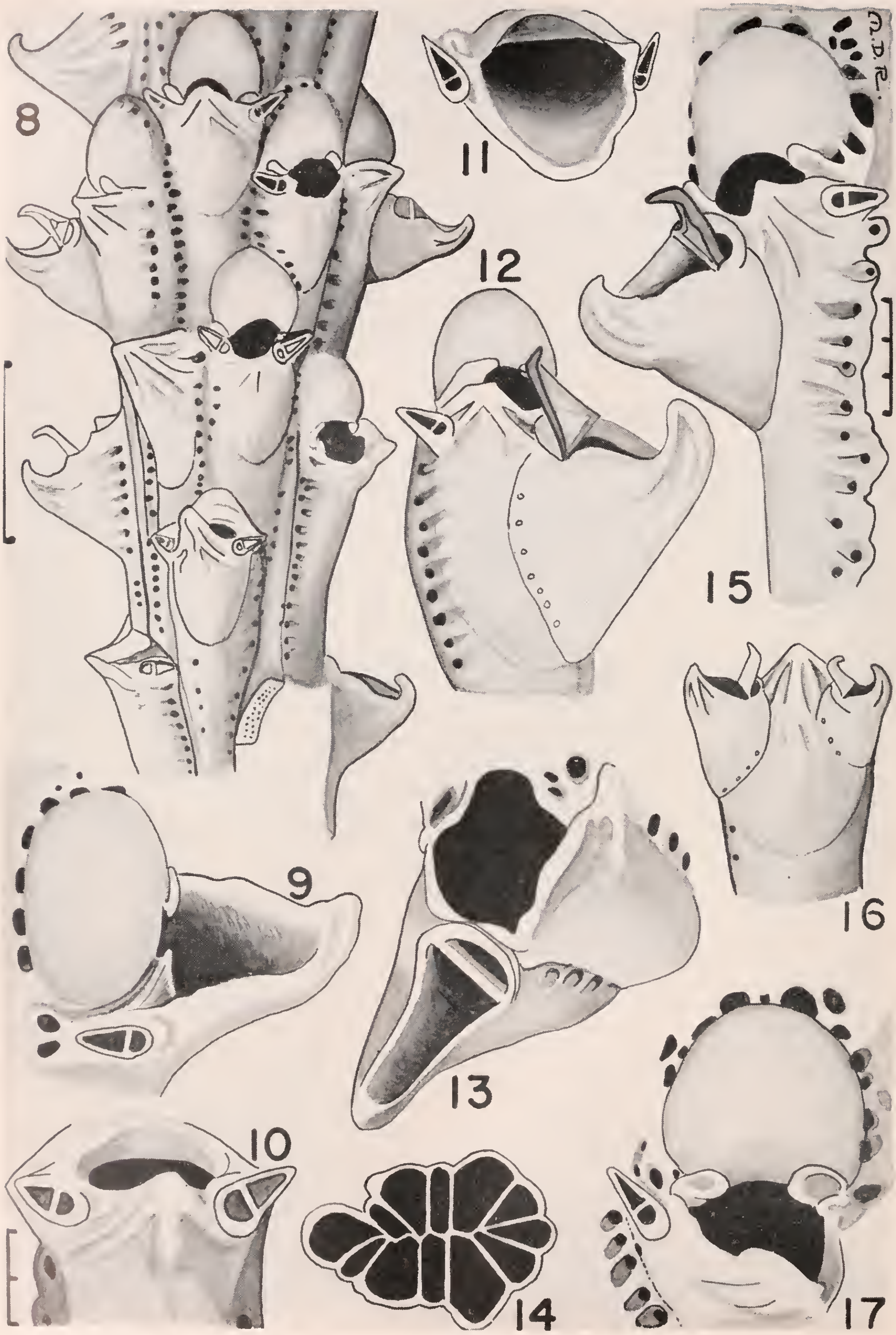
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Plate II

shorter, chitinous band around the lucida. The mandible tip is a scalloped membrane turning inward into the beak, somewhat as in *Escharoides praestita.* The mandible is shaped like a thumbnail or like the side view of a thimble. Four or five small pores penetrate the base of the avicularial chamber.

Orifice. The exact frontal boundary of the terminal, subcircular orifice is hard to fix because of the peristome which hides it. Lyrula, cardelles and oral spines are completely absent. A thin semicircular peristome forms an upright collar of uniform height half-way around the poster of the primary orifice. The peristome ends sharply and abruptly at the avicularial beak on each side.

Ovicells. The four ovicells are somewhat salient and partly immersed, imperforate, but outlined with areolar pores. Their surface is smooth to beaded. The peristome does not encroach upon them. Three of the ovicells contain peach-colored embryos, so the species is in active larval production in mid-January.

Distribution and ecology. The holotype came from Sta. 44, in the Wilkes Land area, 65°25′ S. Lat. and 101°13′ E. Long., from 100 fathoms, on Jan. 14, 1948. The paratype came from Sta. 190, Marguerite Bay area (approximately 68°30′ S. Lat. and 68°30′ W. Long.), from a depth of 35 fathoms, on Feb. 20, 1948. The water temperature at time of collection in both places was 30° F.

The holotype was collected right during its larval producing season. The paratype colony is a much younger one than the holotype, judging from its lighter, more

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EXPLANATION OF PLATE II

All figures on this plate are of *Escharoides bubeccata,* n. sp. from Sta. 104, and were made with the aid of a camera lucida.

FIGURE 8. Branch fragment. Three ovicelled zoids have damaged peristomes, hence more of the orifice (large solid black areas) shows than normally would. A proximal wall with numerous pores is diagrammed in the third zoid from left of the bottom row. Drawn to the 1.0 mm. scale at left.

FIGURE 9. Top view of ovicell to show its flattened shape, the two pre-ovicell tabs, the small peristomial avicularium and the extent of the mucro. Drawn to the same scale as Figure 10.

FIGURE 10. The upper part of a zoid with two small peristomial avicularia and the heavily ribbed mucro. Drawn to the 0.2 mm. scale at left.

FIGURE 11. View from above to show the shape of the non-ovicelled zoid orifice. Drawn to the Figure 10 scale.

FIGURE 12. A fairly young ovicelled zoid with a small peristomial and a huge frontal avicularium. The latter is attached directly to the frontal "bib" slightly on the diagonal. Its mandible is open and areolar pores of both zoid and avicularium are noticeable. The peristome is shoved off center, as in Figure 13. Drawn to the Figure 15 scale.

FIGURE 13. Top view of a non-ovicelled zoid showing the distortion of the secondary orifice (large solid black area) and the peristome (ledge to the right) caused by the large frontal avicularium. Drawn to the Figure 10 scale.

FIGURE 14. Cross section through a branch, cutting through the cavities of 12 zooecia. Drawn to the Figure 8 scale.

FIGURE 15. An ovicelled zooecium, older than that of Figure 12. The avicularial areolar pores have been obscured by the thickening of the wall and by age. The mucro is squeezed in asymmetrically between the two avicularia. Drawn to the 0.3 mm. scale at right.

FIGURE 16. A zoid with two medium-sized frontal avicularia. The small peristomial avicularia are absent from this specimen because the frontal ones have replaced them. Mandibles open. The strongly ribbed peak between the avicularia is the mucro. Drawn to the Figure 15 scale.

FIGURE 17. An ovicell with the pre-ovicell tabs in typical position and appearance. Drawn to the Figure 10 scale.

iridescent color and lighter calcification. The holotype colony is penetrated in places by threads of a boring organism and thinly covered with a dusting of diatoms. A few zoids have polypides (soft parts) inside. Sponge spicules are imbedded in some of the zoid bases.

Affinities. This species is somewhat reminiscent of Busk's *Myriozoum marionense* (1884, p. 171, Pl. 23, Fig. 6) which has similar avicularia and orifice but whose frontal is perforated all over and whose growth habit (cylindrical branches) is different.

Escharoides bubeccata, n. sp.

Figures 8–27

Name derivation. The species name, meaning an *Escharoides* provided with huge-beaked avicularia, is derived (with the aid of Dr. R. W. Brown's most helpful lexicon) from these Latin parts: *bu* (huge, monstrous), *beccus* (beak), *ata* (having).

Diagnosis. Colony erect, dendritic; joints chitinized; branching dichotomous. Stalks slightly flattened and serrate, due to the many huge projecting avicularia. Zooecia areolated, thin-walled, greatly attenuated proximally, convex distally. End wall a sieve plate. Side wall with about 4 rosette plates. Orifice terminal, large. Oral spines, lyrula and cardelles absent. Peristome outwardly strongly ribbed, urceolate. Small, long acuminate avicularia (1, 2 or 0) at the two sides of the peristome. A huge avicularium, with massive chamber and strongly hooked, ridged beak, variously placed frontally. A few pores present around the avicularial chamber base. Avicularia adventitious. Mandibles elongate, triangular, with oval lucida and 2 ridges for muscle attachment. Ovicell thin, flattened, globose, hyperstomial, non-porous but bordered by areolae. Ancestrula 6 or 7 spined, the distal pair of spines considerably enlarged.

Measurements.

1.440–3.226	(2.395)	L Non-ovicelled zooecia
0.432–0.518	(0.481)	W Non-ovicelled zooecia
0.360–0.475	(0.432)	L Ovicell
0.360–0.475	(0.436)	W Ovicell
0.230–0.302	(0.278)	L Primary orifice
0.288–0.331	(0.310)	W Primary orifice
0.166–0.216	(0.194)	L Small peristomial avicularium
0.072–0.101	(0.083)	W Small peristomial avicularium
0.072–0.130	(0.102)	L Small peristomial avicularium's mandible
0.058–0.079	(0.066)	W Small peristomial avicularium's mandible
0.346–0.605	(0.490)	L, at top, of huge frontal avicularium
0.202–0.288	(0.245)	W, at top, of huge frontal avicularium
0.576–0.763	(0.673)	Height of huge avicularium's attached surface
0.202–0.389	(0.311)	L Mandible of huge frontal avicularium
0.158–0.288	(0.209)	W Mandible of huge frontal avicularium

Zoarium. The colony takes the form of brittle, yellowish, dichotomously branched, serrated, jointed twigs. The serrations are due to the protruding frontal avicularia and urceolate peristomes. The somewhat chitinized "joints" are yellow to amber color and pinch in a bit. The slightly flattened branches in cross section have up to 14 zooecia (usually fewer) facing outward, all around the branch. The maximum stalk breadth is about 2 mm. The greatest length of any colony fragment was 44 mm.

A young zoarium terminates proximally in a tangle of tubular chitinous rootlets which originate from the zoids beyond the ancestrula and also from zoids higher up, near the joints. These rootlets arise most inconspicuously. They help anchor the colony to the substratum or to other objects. (Rootlets are shown in Fig. 24.)

There are about two ounces of colony fragments in the collection.

Ancestrula. No embryos were present in the ovicells. The 8 ancestrulae (or parts of ancestrulae) found attached to the bases of young colonies were already devoid of polypides, indicating that embryo release and metamorphosis must have taken place quite some time before the date of collection, Jan. 29, 1948.

The ancestrula is quite different in appearance from the rest of the colony (Figs. 18, 23, 25). It resembles a *Chaperia* zoid in having a large membranous area over which 6 or 7 stout spines gently curve. The most distal pair of spines is thicker, longer and may flatten out to branch further, like antlers. The ancestrular front around the membranous area is thin, imperforate, smooth, hyaline.

Zooecia. Zooecia somewhat clavate, especially narrow and attenuated proximally, terminate like a wedge back of the next proximal zoid in its linear series. The distal part of the zooecium is expanded due to the urceolate peristome and the peristomial avicularia. Zooecial walls are very thin and hyaline. The frontal wall is areolated, smooth except for the thickened ridges which lead up to the peristome and avicularial beaks. The areolar pores become smaller and rounder at the point where they actually puncture the frontal wall. Four multiporous rosette plates were counted in each lateral wall of one zoid. The proximal wall is a slanting sieve plate (Fig. 8) with numerous small pores (about 16, more or less) sometimes arranged in two rows, other times more irregularly scattered.

The polypides are very long, befitting such long zooecia.

Avicularia. The number of avicularia per zoid may range from 0 to 1 or 2. Avicularia vary in size but there are two major types: Type I (peristomial), Type II (frontal). Type I is small, long, slender, sharply acuminate and always located at the orifice corners, near the start of the peristome. It is directed obliquely backward or laterally (Figs. 9, 10, 11). Type II is the more variable as to position and size but is considerably larger than Type I (Figs. 12, 13, 15, 16). Type II occurs anywhere on the swollen part of the zooecium, on the peristome itself or along one side of it, sometimes slightly on the diagonal. Both the large frontal and the small acuminate peristomial avicularia are perched upon an avicularial chamber, the former on a very large one which contains 2 massive bundles of mandibular muscles. Both avicularial chambers are bordered by a few tiny areolar pores. Type II avicularia have a large, powerfully hooked beak whose curved tip is reinforced with several converging thickened ridges. Such ridges are lacking in Type I avicularia, but the beak tip may be slightly hooked. Type II avicularia vary considerably in size, some being about double the height of others. All avicularia point outward, away from the polypides. Mandibles of both types are very similar in shape and general plan (Figs. 19, 27). Both are triangular, with a hooked tip and oval lucida and two reinforced converging bars for the attachment of mandibular muscle tendons. Type I mandible is only lightly chitinized, Type II heavily so.

Such massive avicularia are unusual. One wonders what role they play in the activities of the colony. Calvet (1931, p. 107, Pl. 2, Fig. 16) pictured similar huge avicularia in *Schizellozoon dentatum.*

Polypides. The polypides (tentacles and gut) are very long, befitting such long zooecia. About 12 tentacles were counted on a very young everted zoid.

Orifice. The terminal orifice (Figs. 11, 13) is similar to those of *E. praestita* and *E. tridens.* The anter and poster are both convex but the poster is considerably larger. In ovicelligerous zoids there are two tabs or small plates, one on each side, just in front of the ovicell (Figs. 9, 17). Lyrula, cardelles and oral spines are absent. An urceolate or platform-like strongly ribbed peristome juts out in front of the orifice (Figs. 9, 10).

Ovicells. The thin-walled ovicells are smooth, imperforate, salient though slightly flattened, gently mitre-shaped to globose and outlined by areolar pores (Figs. 8, 9, 15). The areolae are formed by the encroachment of the neighboring zoid's frontal wall. All ovicells were empty, *i.e.,* without embryos, at the time of collection.

Distribution and ecology. This species was dredged from Antarctic Sta. 104 and 190, mostly from the former. Station 104 was off Cape Royds, Ross Island, at 58 fathoms, Jan. 29, 1948, a considerable distance away from Sta. 190. Relatively few forms are found growing on or attached to living colonies of *E. bubeccata:* —calcareous worm tubes, hydroid stalks and *Notoplites tenuis* (bryozoan) zoids. On dead stalks can be found additional materials: foraminiferan shells, folliculinids, sponge spicules, diatoms, *Caberea darwinii, Hippothoa bougainvillei* and cyclostomatous bryozoa.

Escharoides praestita (Waters) 1904

Figures 28–38

Synonymy.

1904. *Smittia praestita.* Waters, pp. 67–68; Pl. 8, Figs. 10a, b. Lat. 70°23′ S., Long. 82°47′ W., at 480 meters. Two specimens.

Discussion. This species, and others of this paper, have been removed to *Escharoides* from *Smittia (Smittina)* because they lack the lyrula and cardelles characteristic of the Smittinidae.

Waters's original description was made from only two available specimens, apparently rather young and lightly calcified, and the species has not been reported since, till the present time. Therefore, it is possible to add considerably to his brief but excellent original description. The U.S.N. specimens fit Waters's illustrations better than his description. The chief points of conflict between them and Waters's description (outside the fact that Waters's specimens were possibly very young) are the presence of areolar pores outlining, but not perforating the ovicells and some inconsistencies regarding the avicularial and mandibular shape. Waters (p. 67) describes the ovicell as "not areolated" and the avicularia as "round . . . with a semicircular mandible" but pictures the avicularia as elliptical and with a mandible that is a bit longer than wide.

Diagnosis. Zoarium encrusting. Pleurocyst areolate. Frontal convex, converging toward the primary orifice then diverging again into an urceolate peristome. Conspicuous oral spines, four in all autozooecia, two in all ovicelled zooecia. Ovicells globose, non-porous but outlined by areolae. Oval avicularia, if present, are placed high up at either or both sides of the peristome. Beak oriented laterally to distally. Mandible shaped like side view of a thimble. Orifice poster a wider curve than the anter. Both convex. Lyrula and cardelles absent. Four or five dietellae.

Measurements.

0.936–1.483	(1.115)	L Zooecia
0.720–0.864	(0.781)	W Zooecia
0.137–0.187	(0.159)	L Avicularia
0.101–0.122	(0.115)	W Avicularia
0.043–0.058	(0.049)	L Back area of avicularium
0.101–0.112	(0.113)	W Back area of avicularium
0.166–0.202	(0.184)	L Primary orifice
0.216–0.288	(0.274)	W Primary orifice
0.331–0.418	(0.367)	L Ovicells
0.403–0.518	(0.467)	W Ovicells
0.072–0.144	(0.105)	L Mandible
0.065–0.115	(0.088)	W Mandible
0.029–0.058	(0.045)	Diameter of spines

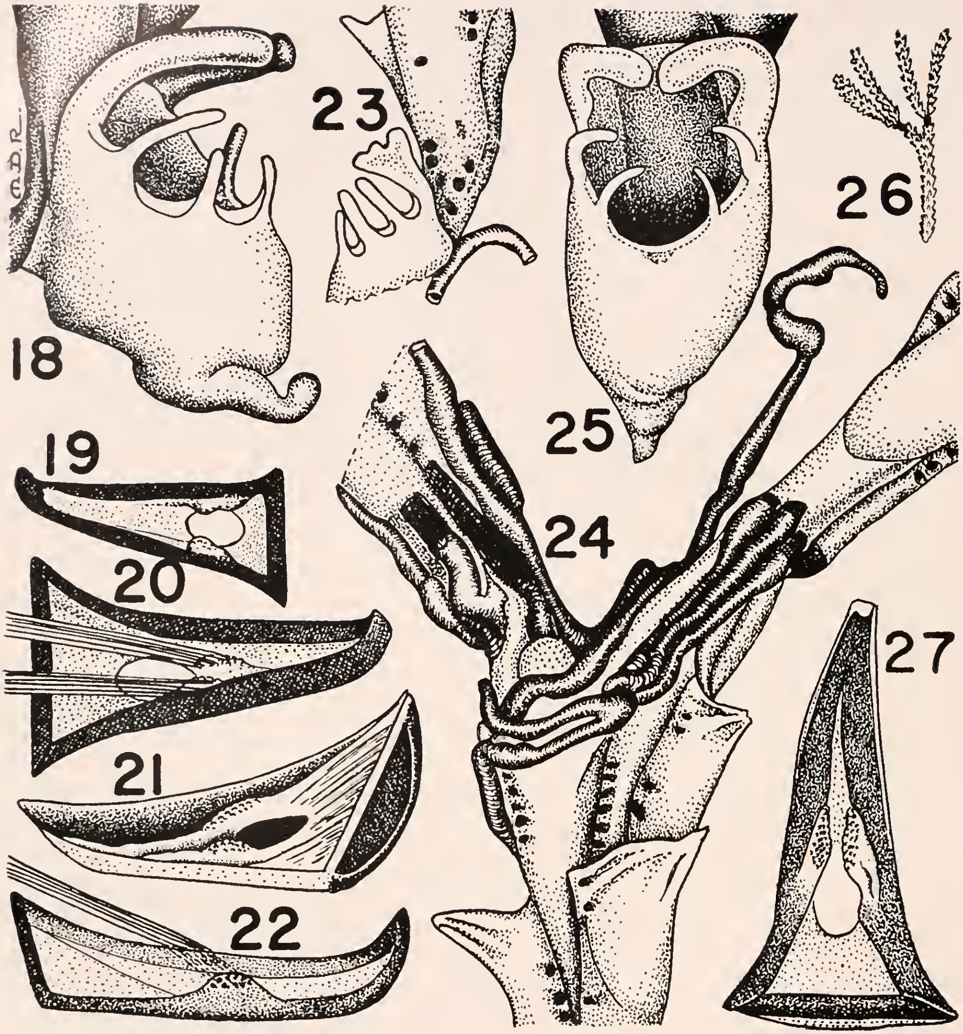
Zoarium. Coarse, whitish, well-calcified, unilaminate colonies encrusting stones and other bryozoa.

Zooecia. Zooecia large, hexagonal, well-defined. Mural rims not salient. Frontal an areolate, granular pleurocyst of varying thickness, thinnest medially and thickest at the urceolate peristome (Figs. 28, 32). Frontal convex, gradually converging to the primary orifice, then abruptly diverging into the peristome (Figs. 28, 29, 31, 37). This convergence-divergence is one of the several distinguishing features between *E. praestita* and *E. tridens.* The areolar pores are rather small, arranged in one or two rows, reminiscent of *Mucronella crozetensis* (Fig. 29). Small costulae may develop on some zoids.

Four or five dietellae appear on the zooecial basal surface. The zooecial walls curve in basal aspect. The three more distal walls are convex, the three proximal concave.

Avicularia. One or two medium-sized oval avicularia may occur on some zooecia, ovicelled or non-ovicelled. They are always located on the shoulders, against the corners of the peristomial collar, on a slight elevation or small avicularial chamber which is marked off from the rest of the frontal by a few (two or three) very tiny pores. This chamber tips the beak upward a bit, to face the observer. Mandible slightly longer than wide, shaped like the side view of a thimble, reinforced around the edge by a broad chitinous band, and some distance in from the edge by a second band. A large rounded lucida is present.

Orifice. Nearly terminal. Its poster is protected by an extensive urceolate peristome. Its anter is outlined by a spine-bearing ledge. Both anter and poster



Explanation of Plate III

All figures on this plate are of *Escharoides bubeccata*, n. sp., and are drawn with the aid of a camera lucida unless otherwise specified.

Figure 18. Side view of a very young lightly calcified ancestrula. A rootlet is at the extreme left, breaking away from the succeeding zoid.

Figure 19. Mandible of a small peristomial avicularium.

Figure 20. Mandible of a huge frontal avicularium with the two tendon bundles attached.

Figure 21. Mandible of a large frontal avicularium showing its inner surface and articulating end. Lucida is left black.

Figure 22. Exact side view of a large frontal avicularial mandible with tendon attached to the inner raised ledge.

Figure 23. Another older ancestrula more strongly calcified than that of Figure 18. Also, it has 7 spines, with the most distal pair antlered. The basal part of the ancestrula has been broken away or damaged. The curved tube to the right is part of a rootlet.

are convexly curved, the poster in a wide arc. Constant, conspicuous, hollow oral spines occur, four in non-ovicelled and two in ovicelled zoids. The outer pair is much bigger, heavier and thicker-walled than the inner, more distal pair of spines. Both pairs are "jointed" at the base. The bases of the outer pair jut into the orifice to some extent while the bases of the inner pair are some distance back of the orifice. The outer pair marks the terminus of the peristome. Lyrula and cardelles are absent. Opercula are delicate, their boundaries hard to define.

Ovicells. Ovicells hyperstomial, globose, usually very salient but sometimes partly imbedded in the next distal zooecium (depending upon age of colony, degree of calcification and amount of crowding). Ovicell surface smooth to granular, nonporous, although small areolar pores occur around it where it touches the next distal zooecium, but these areolae do not perforate the ovicell proper, only outline it. The peristome does not encroach upon it. The two strong spines occur at its sides, between it and the peristome.

Distribution and ecology. This species has been reported only once before, by Waters, from the Antarctic. The U.S.N. specimens came from Antarctic stations Nos. 44 and 240, and from pebbles Nos. 2, 7 and 10. Station 240 was at Marguerite Bay (approximately 68°30′ S. Lat. and 68°30′ W. Long.), at a depth of 40 fathoms, water temperature 30° F., Feb. 22, 1948. The pebbles, Nos. 2, 7, 10, were from a small lot of material to which no collection data were appended.

The Sta. 44 *E. praestita* grew on a *Smittina*. The other station material was on rocks. Some colonies from Sta. 240 were partly overgrown by a colony of *Microporella,* by sponge debris and pink coralline algal crust.

Affinities. Escharoides praestita closely resembles *E. jacksoni* (Waters) as pictured by Osburn (1953, p. 780, Pl. 81, Fig. 8). The latter differs from *E. praestita* in having perforated ovicells and bigger, sharply pointed avicularia.

Escharoides tridens (Calvet) 1909

Figures 39–45

Synonymy.

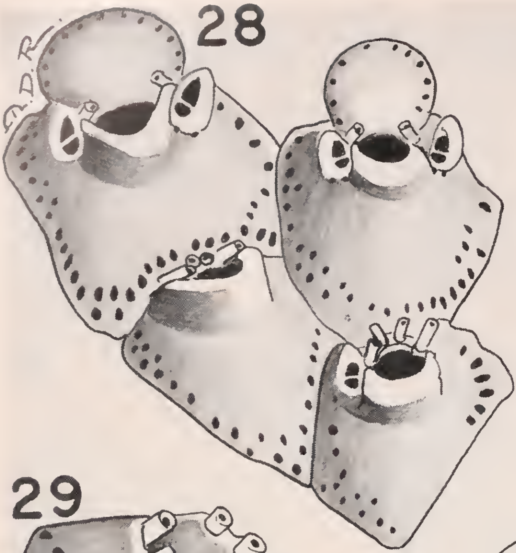
1909. *Smittia praestans* var. *tridens.* Calvet, p. 30, Pl. 3, Fig. 6; from Booth-Wandel and Wyncke Islands and Schollaert Bay, at 30 meters, on algae and gravel.
1928. *Peristomella excavata* var. *tridens.* Livingstone, pp. 7, 8, 61; Commonwealth Bay, Antarctica, 25–30 fathoms, Sept. 3, 4, 1912.
1952. *Smittia praestans* var. *tridens.* Vigeland, p. 9; Sta. 139, Peter I Island, Ranvik Bay, 80 meters (Olstad).
1953. *Escharoides tridens.* Marcus, p. 299.

FIGURE 24. Branch of a colony at point of bifurcation, "joints" and rootlet formation. The dark twisting tubes which spring from the zooecia are rootlets.

FIGURE 25. Frontal view of the Figure 18 ancestrula. The 7th (median) spine apparently has not yet developed.

FIGURE 26. Habit sketch of a colony fragment showing the dichotomous branching and serrate surface. The two darkened pinched-in "joints" are more chitinized areas from which rootlets may develop.

FIGURE 27. Face view of a large, heavily chitinized frontal avicularial mandible.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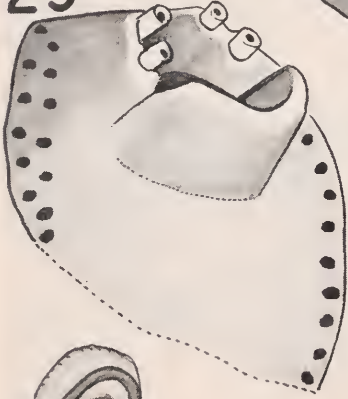
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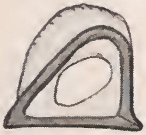
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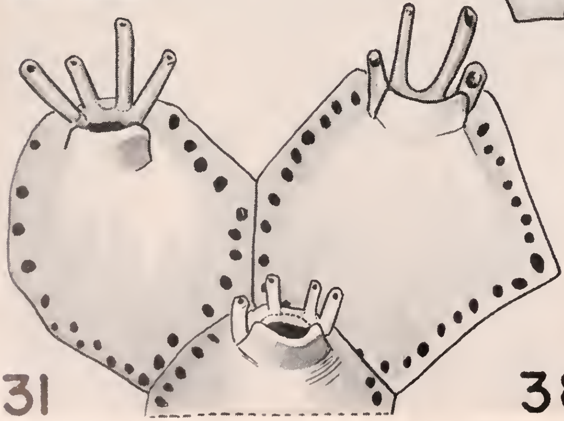
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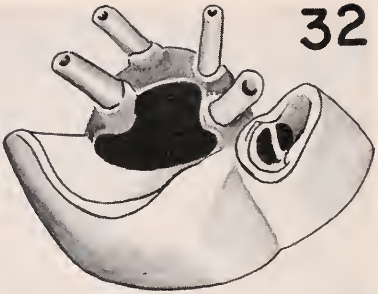
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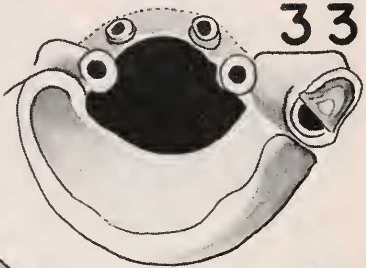
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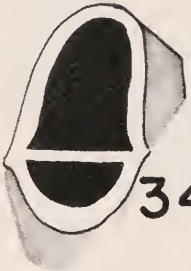
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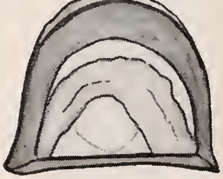
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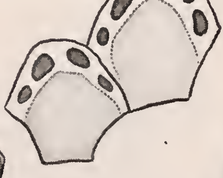
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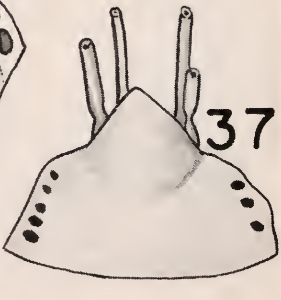
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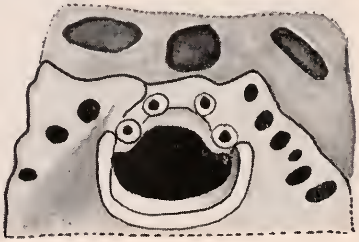
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Plate IV

Diagnosis. Encrusting. Zooecia hexagonal, bracket-like. Pleurocyst areolate. Lyrula and cardelles absent. Primary orifice anter and poster both convexly curved but poster is a much wider arc. Anter limited laterally by two heavy spines. Peristome decidedly urceolate. Areolae outline a small, non-porous, globose ovicell. Oval avicularium peripheral, half way down the zooecial front. Three dietellae.

Measurements.

0.792–1.080	(0.946)	L Zooecia
0.389–0.706	(0.547)	W Zooecia
0.130–0.158	(0.140)	L Avicularia
0.101–0.115	(0.109)	W Avicularia
0.187–0.288	(0.236)	L Primary orifice
0.288–0.446	(0.337)	W Primary orifice
0.245–0.302	(0.269)	L Ovicell
0.331–0.389	(0.366)	W Ovicell
0.043–0.115	(0.075)	Spine diameter
0.065		L Mandible
0.072		W Mandible

Zoarium. White, coarsely patterned, unilaminate, encrusting. Colony looks sturdy. Basal surface fragile and much thinner than frontal.

Zooecia. Hexagonal, distinctly outlined frontally and basally. Mural rims not salient. From the basal aspect the three distal walls are convex, the three proximal ones concave. Three dietellae are visible on some zooecia. The frontal wall is an areolated granular pleurocyst. The underlying olocyst is very thin, smooth and occasionally punctured by the areolar pores. Pleurocyst areolae are round to oval,

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EXPLANATION OF PLATE IV

All figures on this plate are of *Escharoides praestita.* Figures 28, 29, 32, 33 and 34 are from Sta. 44 colonies. Figures 30, 31, 35, 37 and 38 are from Sta. 240 material and Figure 36 is from Pebble No. 10.

FIGURE 28. Four heavily calcified zoids, having 0, 1 or 2 avicularia. Ovicelligerous zoids have two spines, non-ovicelled zoids have four.

FIGURE 29. Urceolate peristome and four oral spines. Zoid without avicularia.

FIGURE 30. A mandible which looks more triangular than it really is because it is seen from an oblique angle.

FIGURE 31. Three very young, lightly calcified zoids, very similar to Waters's figure for the species. Spines, although long, are all broken off at the tip and still slender.

FIGURE 32. Oblique view of a heavily calcified peristome, showing position and orientation of avicularium with respect to the spines and peristome.

FIGURE 33. Top view of a well developed flaring peristome and four broken-off oral spines. The two lower spine bases constrict the primary orifice anter.

FIGURE 34. An avicularium.

FIGURE 35. A heavily chitinized mandible ("thimble-shaped"). The lucida is smaller than in Figure 30 because of the increased chitinization.

FIGURE 36. Free-hand sketch of the basal surface of two zooecia, one with four, the other with five dietellae.

FIGURE 37. Zoid tipped back to show the urceolate peristome from below. The two outer spines show a "joint" (constriction) near their base.

FIGURE 38. A zoid tipped forward to show the oral region and dietellae (the three very large darkened holes above the orifice). No avicularia are on this zoid. Orifice shape typical.

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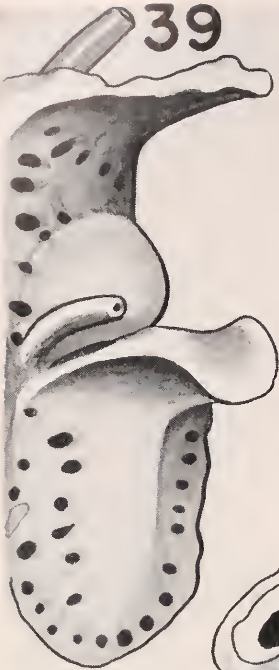

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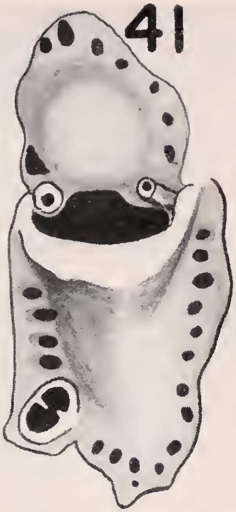

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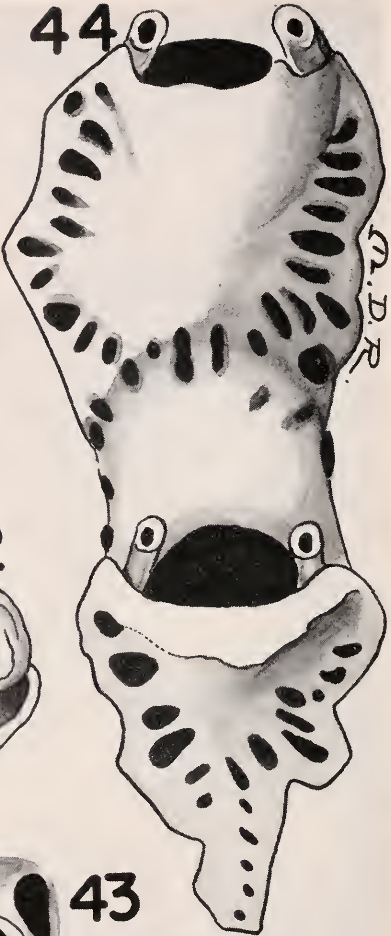

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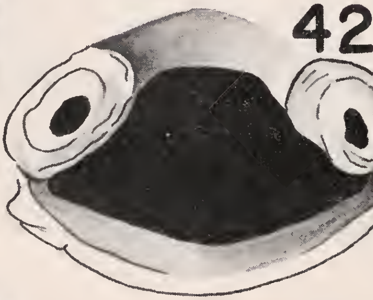

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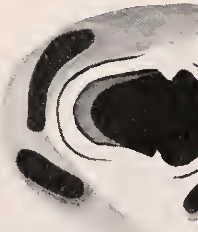

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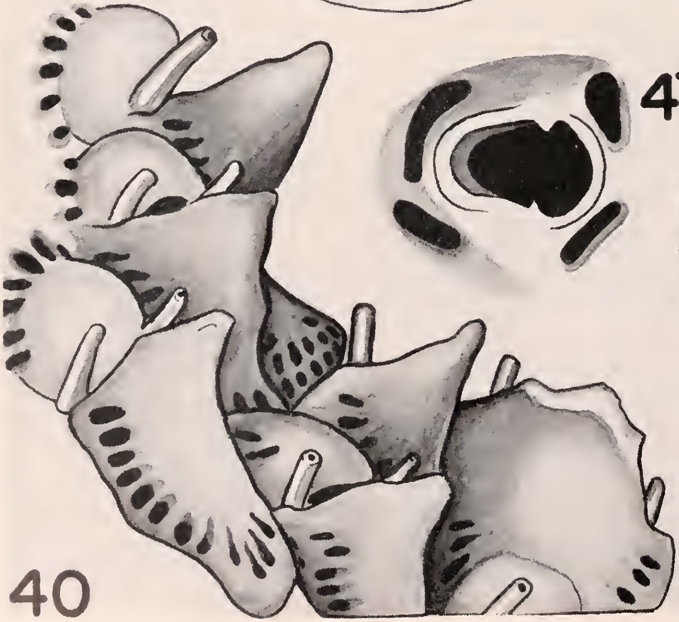

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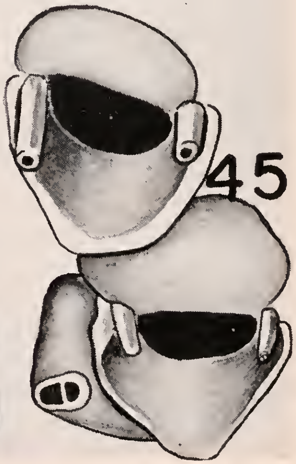

Plate V

variable in size and close together. Interareolar costulae are very narrow and not especially elevated. In occasional very elongate or crowded zoids the proximal frontal may show a latticework of pores where the areolae have become disoriented or added to. Zoological frontal sometimes bulges in the peristomial region like an inverted cone, other times is only gently convex, with the peristome decidedly urceolate (Figs. 39, 40).

Some zooecia bear ovicells, others do not. Some have avicularia, the majority do not (Figs. 41, 44, 45). Two very sturdy oral spines occur at the corners above the peristome and poster (Figs. 42, 45). These spines are hollow, oval in cross section and "jointed" near the base. They occur on all zoids, young or old, ovicelled or non-ovicelled and are usually broken off near the base. Curiously, in this species, these two spines seem to be the most constant and dependable feature.

Polypides are still present in some zooecia.

Avicularia. Zooecia may have one, two or no avicularia. The latter condition is the most frequent. One avicularium per zoid is far more common than two. The avicularia are oval, adventitious, medium-sized and located peripherally near the junction of the distal and proximal lateral walls. Their beaks always point outward and are slightly elevated. Mandible shaped like the side view of a thimble.

Orifice. Proximally (the poster of) the primary orifice is not distinctly marked off and is set fairly deep within the peristome. The anter is smaller and shallower than the poster. Its shape depends upon the extent of the oral spine development, being rather angular if the spine bases are huge, and curved if the spine bases are medium-sized.

The peristome is absent from the distal part of the zooecium but very prominently urceolate frontally, sometimes greatly prolonged, like the lip of a pitcher (Fig. 39).

Lyrula and cardelles are completely absent.

Ovicell. Hyperstomial, salient, rather small, globose to caplike but flattened like a compressed ball. Its wall is smooth to granular, of medium thickness and imperforate although areolar pores outline it. The two thick spines separate it from the peristome.

Distribution and ecology. Escharoides tridens is an Antarctic species. The present study specimens were on rocks and pebbles from Antarctic Sta. Nos. 163

EXPLANATION OF PLATE V

All figures on this plate are of *Escharoides tridens* and are made with the aid of a camera lucida.

FIGURE 39. Two zoids, the lower one ovicelled, with rather flattened frontal and suddenly extended peristomes.

FIGURE 40. Profile view of several zoids whose whole frontal gradually elevates and merges with the extended peristome. The broad zoid at extreme right is seen from below and shows the three points or "denticulations" on the basis of which Calvet erected the species.

FIGURE 41. An ovicelled zoid tipped forward to show the orifice. An avicularium is on the lower frontal, in its typical orientation and location.

FIGURE 42. Primary orifice with the two very heavy, thickened, broken-off oral spines.

FIGURE 43. An avicularium, surrounded by four areolar pores.

FIGURE 44. Two very old short zoids. The broad peristomial orifice is characteristic of this species.

FIGURE 45. Top view of two ovicelled zoids, the lower one with a frontal avicularium which is not peristomial in position but further down on the frontal, as in Figure 41. Areolar pores around the ovicell have been omitted.

and 240. Station 163 material was from a 30-fathom bottom dredge haul "B" at Peter I Island, water temperature 29.6° F. on Feb. 15, 1948, and its *E. tridens* colonies were covered with a dusting of diatoms. One colony grew over a fragment of *Inversiula nutrix* Jullien 1888. Two colonies from Sta. 240 grew into each other but apparently refused to overlap. The colony surface was relatively free of other extraneous growths.

Affinities. Escharoides tridens bears a very strong resemblance to *E. praestita*. Sometimes it is very hard to tell the ovicelled individuals of the two species apart if avicularia are absent. When avicularia are present there is no difficulty. The non-ovicelled zoids can be distinguished by the difference in spine number, four in *tridens,* two in *praestita. Tridens* avicularia are halfway down the side of the body while *praestita* avicularia are near the peristome. The *tridens* front diverges upward to a widely gaping peristome while the *praestita* front converges upward to the peristome, as in a tightened pouch, then diverges outward from the tightened area.

SUMMARY

1. Two new Antarctic species, *E. barica* and *E. bubeccata,* are described.

2. Two additional Antarctic species, *E. praestita* and *E. tridens,* are reported from new localities and considerable morphological material added to the small amount of data known for these two forms.

3. Other bryozoan species incidentally reported here from new Antarctic localities are: *Caberea darwinii* Busk 1884, *Hippothoa bougainvillei* (d'Orbigny) 1839, *Notoplites tenuis* (Kluge) 1914, all from Sta. 104 (Ross Island) and *Inversiula nutrix* Jullien 1888 from Sta. 163 (Peter I Island).

4. Representatives of the *Escharoides* species are on deposit at the Smithsonian Institution, U. S. National Museum.

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COMPARISON OF THE LEVEL OF CHLORIDE REGULATION BY NEREIS DIVERSICOLOR IN DIFFERENT PARTS OF ITS GEOGRAPHICAL RANGE

RALPH I. SMITH

Department of Zoology, University of California, Berkeley 4, Calif.

An ever present problem of comparative physiologists is that of determining whether or not populations from different parts of the geographical range of a species are physiologically equivalent. When differences are noted in physiological performance of presumably similar animals from different geographical areas, it is sometimes easy to assume that the results are attributable to inherent physiological differences, *i.e.*, to the existence of "physiological races." Unfortunately the concept of a physiological race is rarely defined, and the effect of environmental background in determining the results is seldom evaluated. Lack of clarity in this respect characterizes some of the literature on the salinity tolerance and osmotic relations of brackish-water polychaetes, conspicuous among which are the Nereidae, a family with numerous species in brackish and a few even in fresh water. One of the best known euryhaline nereids is *Nereis diversicolor* O. F. Müller. In the literature there is a number of suggestions that physiologically distinct races of this species exist in different regions. Schlieper (1929) showed that *N. diversicolor* from the neighborhood of Kiel possessed marked osmoregulatory ability at salinities of 18‰ and below, and could survive a fortnight in fresh water, although in the latter medium the worms became somewhat swollen. Schlieper's results, combined and recalculated to give the approximate chloride values, are shown in Table I, and have been entered in Figure 7. It is to be noted that Schlieper's values suggest a level of regulation higher than any I have obtained, but since his determinations were of osmotic pressure by depression of the freezing point, they may not be directly comparable with chloride values. In particular, Schlieper's use of body juices pressed from minced worms may have given higher values than would be obtained on cell-free coelomic fluid because of the inclusion of cell constituents and non-electrolytes.

Following Schlieper, Beadle (1931, 1937) studied the mechanisms of adaptation of *N. diversicolor* to lowered salinities. He found (1937) that in a salinity of about 8‰, the body fluids had a concentration equivalent to 10–13‰, indicating an osmotic regulation not so great as that found by Schlieper. Salinity determinations (details not given) in the estuary of the River Blythe in Northumberland, from which Beadle's animals were obtained, indicated that salinities below 20‰ were probably not encountered in nature, and since it was shown that very little regulation took place at salinities above 8‰, Beadle concluded that osmotic regulation as such was not of survival value to the population of *N. diversicolor* in that area. The greater osmoregulatory capacity of the species at Kiel was attributed to the possibility that (p. 67) "the osmotic regulatory mechanism of the animals with which Schlieper worked was better developed as a result of continual subjection to water of lower salinity." In this statement, Beadle does not indicate whether he considered the effect of low salinity to have been upon individual worms during their lifetime and hence an immediate environmental and ontogenetic effect, or whether he considered it to have exercised a selective effect upon successive generations, leading to the development of a physiological race. Ellis (1937) expressed explicitly the latter type of view after finding that *N. diversicolor* from Roscoff, France, showed a much more rapid weight regulation in a salinity of 7‰ than did worms from Plymouth and Bangor in Great Britain. Ellis felt that the results could not be explained on the basis of experimental conditions, and stated (p. 342) that, "It seems reasonable to conclude, therefore, that the physiological difference between the worms from the two geographical positions is racial rather than environmental." This conclusion is accepted by Krogh (1939). It is unfortunate that Ellis gave no data on the actual salinities prevailing in the localities from which his experimental animals had been taken. Only from such information might we deduce whether or not the observed results are explicable on the basis of previous environmental conditioning. A comparable problem is presented by Beadle's report (1937, p. 69), that *N. diversicolor* from the estuary of the River Blythe "showed smaller weight increases in dilute sea water (25 per cent) during the winter than during the summer months." Assuming that 25 per cent sea water represented the same salinity at all seasons, it seems possible that salinities in the Blythe were lower in winter than in summer, and that worms taken in winter were better adapted or prepared for exposure to dilute sea water by their immediate environmental history, and so responded by less swelling than worms taken during the higher salinities of summer. But without knowing the actual pattern of salinity variation in the Blythe Estuary, one may put forward this explanation only tentatively.

TABLE I

Osmoregulation in N. diversicolor from vicinity of Kiel, after Schlieper (1929)

Concentration of medium			Concentration of body juices	
Salinity 0/00	Freezing point depression	Approx. chloride gm./l.	Freezing point depression	Approx. chloride gm./l.
33	—	18.2	Isotonic	18.2
18	−0.95°	9.9	−1.14°	12.0
17	−0.88°	9.4	−1.10°	11.5
8	−0.45°	4.4	−0.86°	8.9
4-	−0.21°	2.2	−0.70°	7.1
Fresh water	(not given)	—	−0.50°	5.0

In the light of the reports mentioned above, a comparative study of *N. diversicolor* in representative parts of its geographical range has been attempted, with special effort to apply a uniform physiological test at each locality and to secure a detailed picture of the salinity regimen which constituted the ecological background of each population. Populations living under three types of salinity have been studied:

1. *Relatively stable high salinity:* Although it was initially hoped that *N. diversicolor* could be found in a fully marine situation, *i.e.,* in a habitat not exposed to any appreciable lowering of salinity, field work at Millport in Scotland and in the region of Plymouth in southwest England failed to reveal any population not subject to permanent or intermittent lowering of salinity (Smith, 1955a, 1956). The most saline habitats of *N. diversicolor* that could be studied were "marine-dominated," with somewhat variable but relatively high salinities. In summary, the ecological findings have been that in marine-dominated situations *N. diversicolor* behaves like a "brackish-water" animal, and is found in the least saline part of the available habitat.

2. *Relatively stable low salinity:* As a contrast to the intertidal marine-dominated environment, the mesohaline waters of the essentially tideless Baltic Sea, where *N. diversicolor* reaches the limit of its range, were of especial interest. The animals of the Baltic have for centuries been subjected to low salinity and have been in nearly complete reproductive isolation from populations exposed to higher or more variable salinities. The point at issue was whether or not this long adaptation had resulted in a distinctive level of regulatory performance. It might be presumed that in an area of prevailing low salinity *N. diversicolor* would have developed superior osmoregulatory powers, and perhaps would have extended its range further into fresh-water streams than its relatives in other regions. On the other hand, it might have responded to permanent low salinity by internal adaptation rather than by increase in regulatory capacity, and might have eased its osmotic problem by adapting at the cellular level to withstand lower salt concentrations in the body fluids. Such animals might then regulate at a lower level than worms from marine-dominated environments. A third possibility is that the osmotic requirements of cells and tissues have remained constant throughout the species, and that the level of chloride regulation in Baltic representatives of *N. diversicolor* does not distinguish them from other members of the species. This problem was studied at Tvärminne, southwestern Finland, where summer salinities are 6‰ or less, and was followed by studies of worms from the Isefjord, Denmark, where a stable upper salinity of about 20‰ is found. The distribution of *N. diversicolor* in respect to salinity is remarkably different in these two areas, a fact attributed (Smith, 1955b) mainly to hydrographic factors.

3. *Varying salinity (estuarine conditions):* Whereas the above habitats present extremes of high or low salinity, the estuarine habitat presents a most complex pattern of salinity *variation.* Studies of this type of habitat have centered in the estuary of the River Tamar, near Plymouth. The general characteristics of estuaries (Bassindale, 1943; Day, 1951; Rochford, 1951) render any exact statement of characteristic salinity almost out of the question. In the present instance, it has been possible to give a general account of the variation in salinity of the *N. diversicolor* habitat in the River Tamar as a function of distance from the sea, intertidal height, and in relation to normal and abnormally heavy fresh-water influx, but not in terms of range and rate of variation within the tidal cycle. The pertinent ecological observations will be found in the paper (Smith, 1956) which logically precedes the present account.

The writer has been fortunate to have had opportunity to carry out combined ecological and physiological studies at the several localities mentioned above; apparently no previous worker has been able thus to work with *N. diversicolor* over so wide an ecological range. The approach to this problem has been to apply to different populations of *N. diversicolor* a standardized physiological test by as nearly identical methods as possible. The most obvious difficulty in studying the effect of an environmental variable such as salinity is that if one attempts to compare the responses of animals in, say, a salinity of 5‰, this salinity may represent practically normal conditions to a Baltic worm, but a drastically diluted environment to a worm from a marine-dominated habitat. Furthermore, in adapting worms from any locality to a series of test salinities, the length of time spent in adapting the test animal varies according to how close the experimental salinity is to that of the natural waters of the locality. Thus, in attempting to determine whether populations of *N. diversicolor* in different regions are physiologically distinct in respect to salinity tolerance, we must admit at the outset that even if identical test salinities are used in the laboratory, the adaptation to these test media is possibly a much more difficult process in the case of a worm from a habitat of high salinity than it may be for a worm from mesohaline waters. Although most previous studies of the responses of *N. diversicolor* have involved an abrupt change of salinity, an osmotic emergency to which the worm must respond, I have chosen to study this species in a state of as complete as possible adaptation to experimentally lowered salinity. I do not wish to minimize the importance of the emergency response, for this may indeed be crucial in determining survival in areas of varying salinity, but have felt that a study of the fully adapted condition would be less influenced by the environmental history of the experimental animals.

Material and Methods

1. *Experimental animals.* Worms of 150 to 250 mg. in weight were most commonly used; the size was a matter of convenience in maintenance and sampling, since no differences in chloride regulation as a function of size were found. Following collection, animals were sorted into pans containing a layer of washed sand, preferably from the site of collection (at times, especially in the Baltic, worms were reluctant to burrow in foreign sand), and were allowed to establish themselves in burrows. Injured worms and those unable to burrow were discarded; throughout this study the ability of a worm to re-establish a burrow in sand was used as a test of normality, and worms unable to burrow were considered unfit for experiment. Worms from a given collection were distributed randomly into a series of 4–8 bowls, with sand, each containing 2–6 worms, depending upon size. After another day or two, stepwise dilution or concentration of the several media was started. Ordinarily each step changed the concentration by not more than ⅓ of the previous value. It was intended to avoid "shock effects" which, as Wells and Ledingham (1940) have pointed out, might be expected if the salt concentration of tissue fluids changed too rapidly. In changing media, the previous medium was gently decanted, so as not to dislodge worms from their galleries in the sand, and fresh medium added. This method afforded a certain protection to the worms, since they usually stayed in their burrows in sand whose interstitial salinity was only gradually brought to the new value. Ordinarily the worms re-opened their burrow entrances a few minutes after the disturbance and resumed irrigation. Media were changed at 2–4 day intervals, and worms were fed about equally often with finely-minced

flesh of *Mytilus*. Worms were maintained in vigorous condition up to three months in the laboratory, at room temperatures varying from 12–18° C. in winter in Britain to 20–24° C. in the Baltic region in summer. To the possible criticism that the animals were under "laboratory" rather than "natural" conditions, it may be said that the method ensured that experimental animals did not merely "survive," but "lived" in the laboratory, as evidenced by normal burrowing, irrigating, and feeding activity, and that the use of specimens damaged in collection was avoided. When a bowl of worms had been brought to the desired salinity, it was held stable for 5 or more days, the medium being changed twice at the final salinity before the worms were tested. Dilution of media was by tap or lake water (Glasgow), tap water (Plymouth and Copenhagen), or well water (Tvärminne), and these were used as the final medium of lowest salinity at the latter three places. In all cases these were "soft" waters (low in Ca) and, with the possible exception of Glasgow tap water, showed no signs of toxicity. Concentration of media was by the addition of sea water, or by slow evaporation, or, at Tvärminne, by the addition of total sea-salts (kindly given to me by Dr. K. J. Purasjoki).

2. *Taking of samples for analysis.* A worm to be sampled was folded in a piece of cloth wet with the medium and laid upon the lid of a petri dish filled with crushed ice. A few minutes' chilling rendered it sluggish; no other anaesthetic was employed. Coelomic fluid was withdrawn by a Pyrex capillary of about one-mm. bore, drawn out to a tip of 0.1 mm. and fitted to a small bulb of pressure tubing. It was usually necessary to puncture several septa and to move the capillary back and forth in order to secure sufficient coelomic fluid; often blockage of the tip made several punctures necessary. After taking the sample, the pipette tip was sealed in a small flame, and the tube centrifuged to throw down oöcytes and coelomic corpuscles, leaving a clear supernatant of 5–10 mm.^{3} or more. The tube was wiped off with distilled water, the tip containing packed cells nicked with a file and broken off, the tube of clear supernatant set on a small block of plasticene on the stage of a dissecting microscope, and a 5- or 10-mm.^{3} aliquot drawn out by a calibrated micro-pipette. This aliquot was discharged into a previously measured volume of 1–5 ml. of distilled water, giving a dilution of 1 : 100 to 1 : 1000, depending upon the concentration range being tested. Although with a few exceptions worms were used only once, they were returned to their dishes after sampling, and except in occasional instances of obvious injury, promptly dug fresh burrows; in no instances were samples from worms too weak or swollen to burrow used for analysis, since the object of the work was to examine the chloride level in fully and satisfactorily adapted animals. With the technique described it is possible to sample worms as small as 30 mm. in length, and this may be repeated after 8–10 days without apparent ill effect. Samples of media of salinity 9‰ or higher were taken with the same 5- or 10-mm.^{3} pipettes mentioned above, and were diluted in identical fashion. With media of lower concentration, pipettes of 100 or 200 mm.^{3} capacity were used, and samples diluted to 1 : 10 or 1 : 20. With media of chloride content below 0.1 gm./l., samples were analyzed undiluted.

3. *Analytical methods.* This study has been of the chloride content of coelomic fluid as a function of the chloride of the outside medium. Chloride was chosen because it is of the major ions of the body fluid the one least subject to ionic regulation (Robertson, 1949); the equipment for microdetermination was such that it could be transported and used under different conditions with a minimum of variation in

method; and chloride values for the coelomic fluid are conveniently compared with the salinity or chlorinity values in ecological literature. Osmotic pressure would have been fully as acceptable a criterion, but was not attempted for reasons of time and availability of equipment at certain points. It has not been established that chloride concentration parallels osmotic pressure in the coelomic fluid of polychaetes, and it is possible that it may not at low salinities; if this relationship is worked out it may clarify the picture presented here, including the discrepancy between Schlieper's values and mine (see Fig. 7).

Chloride determinations on water samples taken in the field were made by silver nitrate titration, but all determinations on coelomic fluid and on the media to which worms were adapted in the laboratory were by Conway's micro-diffusion method (1950) in one of two forms. In all studies in the autumn and winter of 1953–54 at Glasgow and continuing through June, 1954 at Plymouth, use was made of Gordon's (1952) modification, in which a solution of the dye Fast Green is used as the receptor fluid. Decolorization by chlorine was measured at Glasgow on a Hilger "Spekker" photoelectric absorptiometer with "spectrum orange" (no. 607) filters; at Plymouth a Cambridge Spectrophotometer with wave-length set at 625 $m\mu$ was used. All determinations were in duplicate and averaged. At Tvärminne, where no absorptiometer was available, Conway's (1950) iodometric method was returned to, using an "Agla" syringe burette for titrations. This proved more satisfactory than Gordon's modification, but required 10-$mm.^3$ samples of coelomic fluid at lower salinities. In most instances diffusions had to be carried out at uncontrolled room temperature, and the times of diffusion could not always be held constant. Accordingly, at least four known standards have been used with each lot of unknowns, and a curve established by which the unknowns were estimated. Two separately pipetted and diluted samples of the medium were each analyzed in duplicate at the same time that coelomic fluid chlorides were determined in duplicate on 2–6 worms adapted to that medium. The separate chloride values for the worms have been plotted against the mean chloride values for the media in the figures of this paper; the ranges of the media used are given in the tables. Results have been expressed as chloride in gm. per liter (mg./ml.) of solution; this is nearly equivalent to the "chlorosity" of hydrographers, and is adequate for physiological-ecological work. Approximate salinity values have been obtained by multiplying chloride values by 1.81.

Observations

1. *Marine-dominated populations.* The initial studies of this series were carried out in the Zoology Department of the University of Glasgow on worms collected on the marine-dominated beach at Kames Bay, Millport, the ecological characteristics of which have been described (Smith, 1955a; Watkin, 1942). *N. diversicolor* occupies an upper midtidal zone where the interstitial water of the sand is always rendered somewhat brackish by the subterranean intrusion of fresh water from the land. This zone is considered marine-dominated because it is covered twice daily with pure sea water. Observations made under a variety of weather conditions indicated that the nereid population may in the course of a day have to endure chloride concentrations between 6 and 12 gm./liter (salinities of 11–22‰). However, there is available for about half of each day full strength sea water for burrow

irrigation, so that continuous exposure to salinities below ⅓ that of sea water would be quite outside the range of salinities to which this particular population has been exposed over past generations. In the laboratory two groups of worms were adapted to several different salinities. An initial group collected in the autumn was adapted to dilutions made with Glasgow tap water, which was considered fully satisfactory for general aquarium use. The results (Fig. 1) showed *N. diversicolor* to be isotonic with respect to chloride down to 10 gm./liter and to regulate at lower chlorinities down to 1 gm./1. (a salinity of 2‰) in which body fluid Cl averaged 4.5 gm./1. No evidence of toxicity of tap water was noted during the autumn. However, later difficulties with tap water led to the substitution of Loch Lomond water as the dilutant, and the worms so treated showed a higher level of regulation. The general composition of the Loch water was close to that of tap water, and the improved results are not attributed to increased Ca (shown by Ellis, 1933, to be of great importance in osmoregulation in *Nereis*) since Loch Lomond water is quite soft. Weerekoon (1953) gives the Ca content as 2.8 to 3.3 mg. per liter. It is not clear what may have been the cause of the low level of regulation observed in the autumn. The mortality in the laboratory during the adaptations was heavy, but was the same in the two groups, between ⅓ and ½ of the animals dying during the necessarily long process. The possibility of a seasonal difference in level of chloride regulation cannot be entirely ruled out, nor can the possibility of a slightly toxic effect of the tap water. Since the lower values do not fall below levels observed in other populations of *N. diversicolor* in other regions, it has been felt best to include them in Figure 1, which principally illustrates the results of determinations on the second group of Millport worms, collected in February and tested in late March, 1954. For purposes of recording, media having nearly similar salinities have been grouped. The chloride values for the individual worms have been plotted separately, with mean and standard deviation. It is realized that some groups are too small to permit standard deviation to be used as a measure of significance, but it is used merely to indicate the scatter encountered.

TABLE II

Chloride values, Millport population (spring only)

Chloride of media (range)	Mean cl. of media	Number of worms tested	Mean cl. of body fluid	Std. dev. of body fluid cl.
<0.1 gm./l.	—	—	No survivors	—
0.19–0.50	0.35 gm./l.	14	4.80 gm./l.	0.67
1.02–1.20	1.11	6	6.10	0.65
2.42–2.50	2.47	8	5.94	0.64
5.36–6.20	5.61	22	7.08	0.86
10.35–10.80	10.54	8	11.43	0.45
17.00–20.30	18.65	4	19.15	1.24

It should be noted that I was not able to adapt Millport worms to sea water diluted below a chloride content of about 0.2 gm./1. That at least some of the population would survive at such a concentration is in itself noteworthy, since it represents a salinity of only 0.36‰, about 1.1 per cent of Millport sea water, and far below any salinity this population would encounter in nature.

In comparison with the above data, the results obtained on two other populations from marine-dominated habitats near Plymouth should be noted. Both of the sites to be mentioned experience only very minor lowerings of salinity, but they differ from Millport in representing the marine-dominated end of an extensive habitat of *N. diversicolor* which extends upriver into conditions of extreme salinity variation. The populations studied are each a part of a more or less continuous population which extends into variable estuarine conditions and, in the case of the River Tamar, even into practically fresh water. The first group was collected in May, 1954 at Inswork Point, adjacent to St. John's Lake in the lower estuary of the River Tamar. The results, plotted in Figure 7, indicate a level of chloride regulation little different from that observed with Millport worms in the spring. A second population was sampled near the head of the Kingsbridge Estuary, which

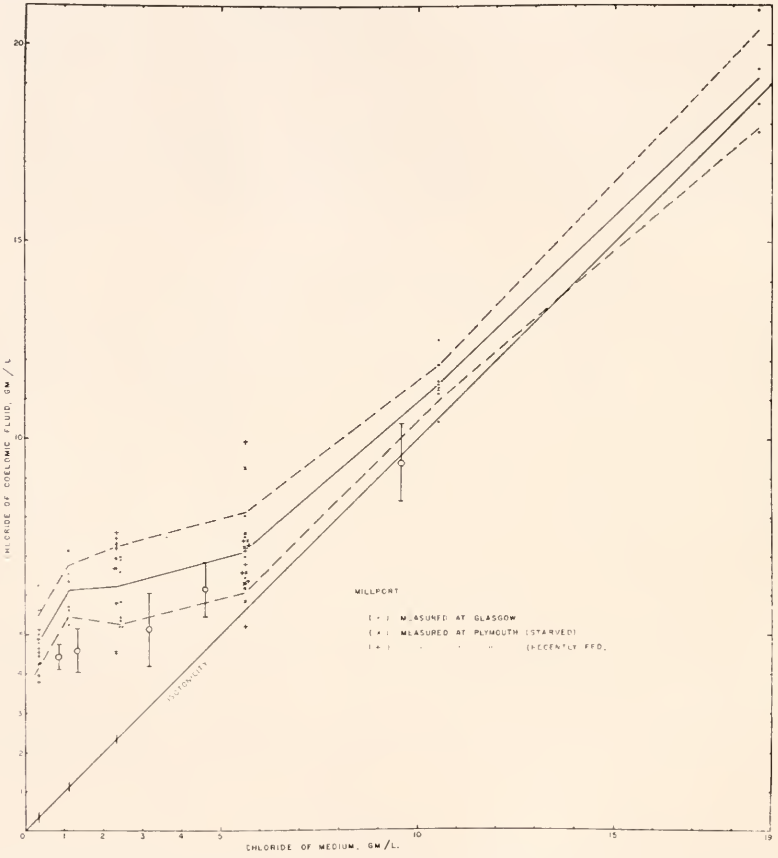

FIGURE 1. Chloride regulation curve of *N. diversicolor* from Millport (spring series; dilutions with Loch Lomond or Plymouth tap water). Solid line, mean; broken lines outline one std. dev. above and below mean. Circles with vertical bars show mean values ± one std. dev. for autumn series (dilutions with Glasgow tap water). "Starved" worms had been without food for two weeks; all other worms fed within 3–4 days of test.

as a whole is marine-dominated. These worms, too, regulated at much the same level as the Millport population (Fig. 2). But it should be noted that it was possible to adapt at least some Kingsbridge worms to fresh water (Plymouth tap water), in which they survived, with feeding and changes of water, for several weeks, at chloride levels of about 0.01 gm./l. To be sure, over half of the worms employed died in the attempt to adapt groups to fresh water, but the fact that some of the population survived in apparently good condition is noteworthy. The actual level of coelomic fluid chloride in worms adapted to fresh water was 2.9 gm./l (std. dev. = 0.59). The general "plateau" of regulation lay higher, at about 6 gm./l. over a range of external chloride up to 4–6 gm./liter. This level of internal chloride should be kept in mind in considering the next population to be discussed, since it is much higher than prevailing chlorinities in the waters of the inner Baltic.

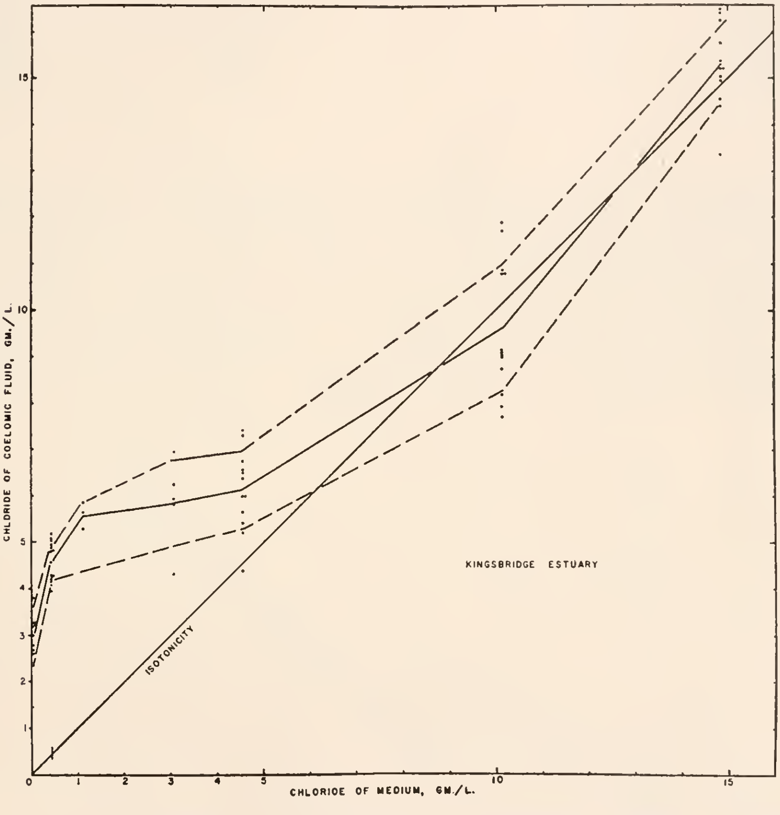

FIGURE 2. Chloride regulation in *N. diversicolor* from Kingsbridge Estuary. Designations as in Figure 1.

2. *Baltic populations.* Studies of *N. diversicolor* near the end of its range in the Gulf of Finland were made at the Tvärminne Zoological Station of Helsinki University. At Tvärminne, *N. diversicolor* is found in shallow waters which in summer have a salinity close to 6‰ (chloride = 3.3 gm./l.), decreasing to 4‰

TABLE III

Chloride values, Tvärminne populations

Chloride of media (range)	Mean cl. of media	Number of worms tested	Mean cl. of body fluid	Std. dev. of body fluid cl.
0.01–0.09	0.03 gm./l.	23	3.35 gm./l.	0.58
0.23–0.43	0.31	21	3.83	0.66
0.81–1.11	0.93	12	4.56	0.64
1.71–2.50	2.03	18	4.86	0.61
3.13–3.92	3.48	16	5.15	0.54
4.20–4.90	4.60	6	5.34	0.59
5.55–6.75	6.17	12	6.03	0.58
7.55–7.75	7.65	5	7.18	0.15
9.80–10.98	10.39	5	9.50	1.41

(chloride = 2.2 gm./l.) at the inshore limit of the ecological range in the local salinity gradient. For present purposes it is important to note that although the waters of the inner Baltic are free of tidal salinity fluctuations, they are subjected annually to drastic lowering of salinity at the spring thaw. This presents certain unique problems and may be the crucial factor in checking the extension of the range of *N. diversicolor* into lower salinities. It is a striking fact that in the inner Baltic *N. diversicolor* seems to be limited at summer salinities of not less than 4‰, even though a number of associated brackish-water species, which in Britain and

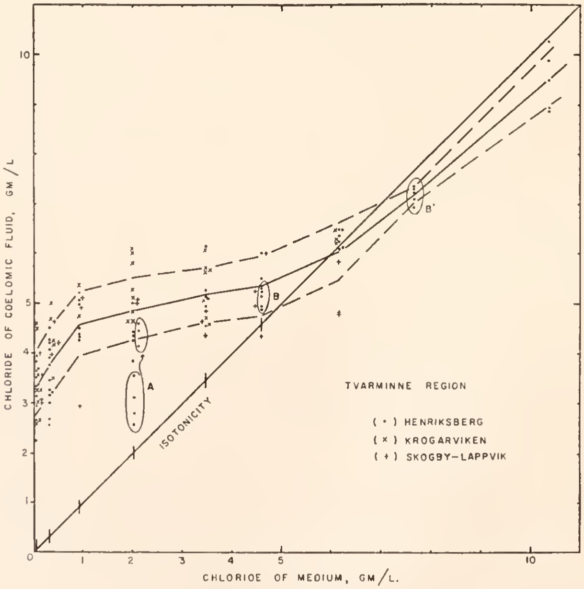

FIGURE 3. Chloride regulation in three populations of *N. diversicolor* from the vicinity of Tvärminne, southern Finland. Encircled groups of points indicate groups retested after two weeks; group A (inexplicably low) fell into line upon retest, group B was retested at a higher salinity at B′. Initial results on group A omitted from calculations.

Denmark do not extend so far into estuaries as does *N. diversicolor,* here penetrate further into less saline waters.

Tests of the level of coelomic chloride were made on groups of worms from three localities, the relative positions of which are shown in a previous paper (Smith, 1955b). Henriksberg represents a habitat adjacent to the open Gulf and probably not exposed to much local fresh-water drainage in the spring; the Krogarvik is a small bay close by the Zoological Station, shallow and so situated that it must receive fresh-water drainage from the land; the shores between Lappvik and Skogby experience a reduced salinity even in summer and lie close to the inshore limit of *N. diversicolor.* Since no differences in the level of coelomic chloride regulation have been observed between worms from these three localities, the results have been combined in the following tabulation (Table III) and in Figure 3.

Worms from anywhere in the Tvärminne area tolerated soft fresh water without noticeable swelling or other ill effects. Regulation of chloride occurs in media of chloride concentration below 5 gm./l., equivalent to a salinity of 9‰. Worms sampled immediately after collection from the Krogarvik are included in the group adapted to media in the chloride range of 3.13–3.92 gm. It is clear that *N. diversicolor* is regulating in nature in summer, even though the chloride concentration of the local water is equal to the internal chloride concentration of worms regulating in fresh water in the laboratory. Thus chloride regulation is carried on when it might appear physiologically unnecessary. It must be kept in mind, however, that the above results were obtained at summer temperatures (15–18° C. in the local waters, 20–22° C. in the laboratory). Regulatory ability is doubtless more crucial in winter when metabolism may be lowered by cold, and even more so during the spring thaw when fresh water from melting snow and ice lowers the salinity of the shallow waters. Segerstråle (1951) has reported that lowering of salinity to less than half of the annual mean salinity occurred in three years out of four in the Krogarvik during the period 1929–1932. Salinities as low as 2‰ and even 0.21‰ (chlorides of 1.1 and 0.11 gm./l.) have been recorded at the bottom of the Krogarvik at such periods of low salinity, and these may persist for several weeks in the spring (February–May). There is thus an obvious need for regulatory ability even if this is not essential in summer. Salinities above 7‰ (Cl = 3.9 gm./l.) are certainly not encountered in nature in the Tvärminne area. In the experimental adaptations there has been noted a tendency to hypotonicity of chloride in media with chloride concentrations greater than 5 gm./l.; this is of questionable significance because of the small number of animals studied. It possibly indicates that total osmotic regulation involves substances in addition to the salts which are represented by the chloride values. Hypotonicity of chloride is not in itself proof of hypo-osmotic regulation.

Following the studies at Tvärminne, work was continued at the Zoophysiological Laboratory, Copenhagen, using *N. diversicolor* collected below the outlet of a stream flowing into Vellerup Vig, a small bay on the west shore of the Isefjord, Zealand, Denmark. The ecological conditions here have been reviewed (Smith, 1955), and it is necessary to note only that the general salinity of the Isefjord is close to 20‰ (chloride = 11.1 gm./l.), that the salinity is quite stable and not disturbed by a great spring lowering comparable to that characteristic of the Gulf of Finland, and that there is a small tidal fluctuation in water level and in salinity. Conditions are thus nearly midway between those in British marine waters and in the inner Baltic. In Vellerup Vig, *N. diversicolor* showed a pattern of distribution unlike that in Finnish waters, and was found even at the mouths of streams where salinities of less than 1‰ persisted for days at a time in the rainy summer of 1954, and where the worms were beneath flowing fresh water for part of each tidal cycle unless relieved by a high stand of water caused by winds. The experimental results, summarized in Table IV and plotted in Figure 4, were obtained by methods very nearly identical to those used at Tvärminne. Laboratory temperatures were somewhat higher (20–24° C.).

The similarity of the level of chloride regulation of *N. diversicolor* from the Isefjord to that of worms from Finland is evident. The differences in the distribution of these populations relative to the local salinity gradient would seem to be the result of ecological factors acting upon some aspect of the life history, or of some physiological factor other than the level of chloride regulation in the well-adapted state.

TABLE IV

Chloride values, Isefjord population

Chloride of media (range)	Mean cl. of media	Number of worms tested	Mean cl. of body fluid	Std. dev. of body fluid cl.
0.02	0.02 gm./l.	12	3.59 gm./l.	0.51
0.39–0.43	0.41	11	4.29	0.53
1.79–1.98	1.91	11	4.97	0.33
4.02–4.50	4.27	11	5.74	0.25
9.28–9.60	9.47	10	8.94	0.36
14.90–15.15	15.03	6	15.02	0.62

3. *Estuarine populations:* Owing to the complexity of the salinity pattern in a typical estuary and the great differences which exist not only between different estuaries but between different parts of the same estuary, it was not possible to select one collecting site "typical" of estuarine conditions. The approach was to attempt to depict salinity conditions in one rather typical estuary, that of the River Tamar, and to select populations for physiological study from well-defined parts of that estuary. One such population from the lower part of the Tamar and another from the Kingsbridge Estuary have been discussed above in connection with "marine-dominated populations." There remains to be discussed the population of *N. diversicolor* from the upper reaches of the Tamar Estuary. According to field observations (Smith, 1956), the Tamar can be divided into three salinity zones, one being the marine-dominated lower reaches where salinities are almost always high; a middle zone where variation in salinity is the predominating feature, with a considerable variation in "mean" salinity both seasonally and with intertidal height; and the upper reaches, where salinities are usually low and where the magnitude of variation is correspondingly reduced. The population of *N. diversicolor* selected for contrast with a marine-dominated population was collected chiefly from Cotehele Quay, with additional material from North Hooe downstream and Calstock upstream. North Hooe lies close to the upstream end of the middle portion of the estuary; salinity variation both intertidally and seasonally is large; the site has the highest density of *N. diversicolor* observed in this study and in the studies of Spooner and Moore

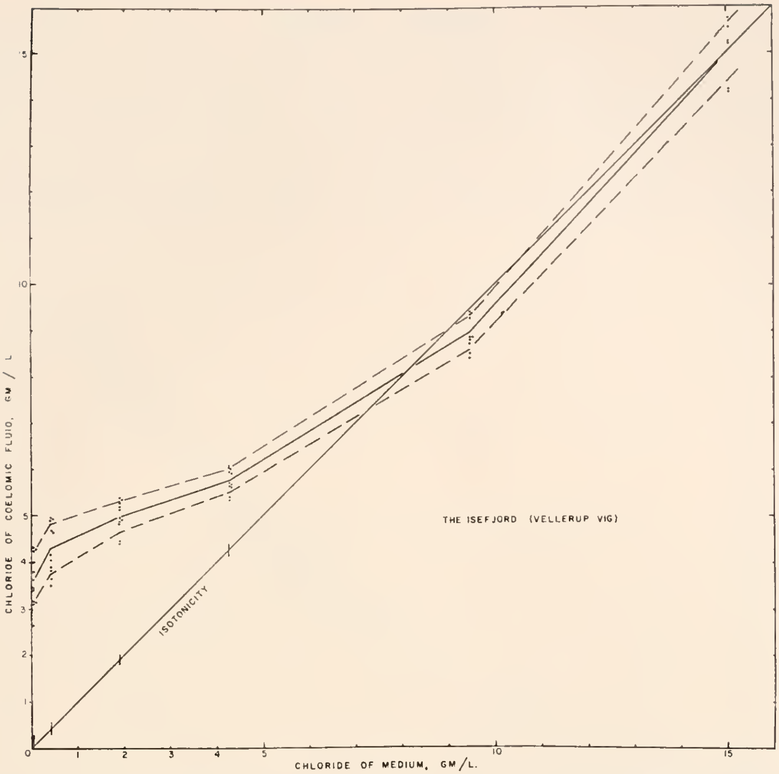

FIGURE 4. Chloride regulation in *N. diversicolor* from the Isefjord, Denmark. These animals were collected at the same site as those studied by Jørgensen and Dales (in press).

(1940). Cotehele Quay experiences low salinities at all times (no over 3‰) in 1954; the nereid population is less than at the preceding station but still exceeds 600 per m^2. At Calstock, *N. diversicolor* reaches its upstream limit; salinities in 1954 may not have exceeded 0.5‰; the worms are few and scattered. Since the results obtained on worms from all three localities are essentially alike they have been grouped in Table V and Figure 5.

TABLE V

Upper Tamar Estuary; combined groups from North Hooe, Cotehele Quay, and Calstock

Chloride of media (range)	Mean cl. of media	Number of worms tested	Mean cl. of body fluid	Std. dev. of body fluid cl.
0.01–0.16	0.07 gm./l.	24	3.38 gm./l.	0.83
0.20–0.48	0.35	28	4.19	0.61
0.63–1.10	0.92	8	5.13	0.47
1.77–2.58	2.04	20	5.03	0.69
3.90–5.13	4.61	15	6.12	0.41
8.32–11.20	9.77	16	9.40	0.53
13.36–14.08	13.76	10	14.36	0.71

4. *Temperature and other experimental variables.* The level of chloride regulation in the upriver Tamar population does not differ from that of Tvärminne or Isefjord worms. But before accepting this apparent similarity, or evaluating the over-all results of the physiological tests, several possible sources of error should be discussed. In this study it was not possible to avoid the fact that worms from the different localities had to be studied at different times of year; however, no clear seasonal trends have been noted. The results obtained on Millport worms are ambiguous in this respect. The stage of development of oöcytes in the coelomic fluid has varied, but since no collecting was done during the actual spawning period and no recognizable males or weakened post-spawning females were subjected to tests, it is hoped that possible effects of the breeding cycle upon the level of chloride regulation have been minimal. As a further indication that seasonal factors have not seriously altered the picture of chloride regulation, the results obtained on 37 worms from the upper reaches of the Tamar in the spring did not differ significantly from those obtained on 84 worms in the autumn.

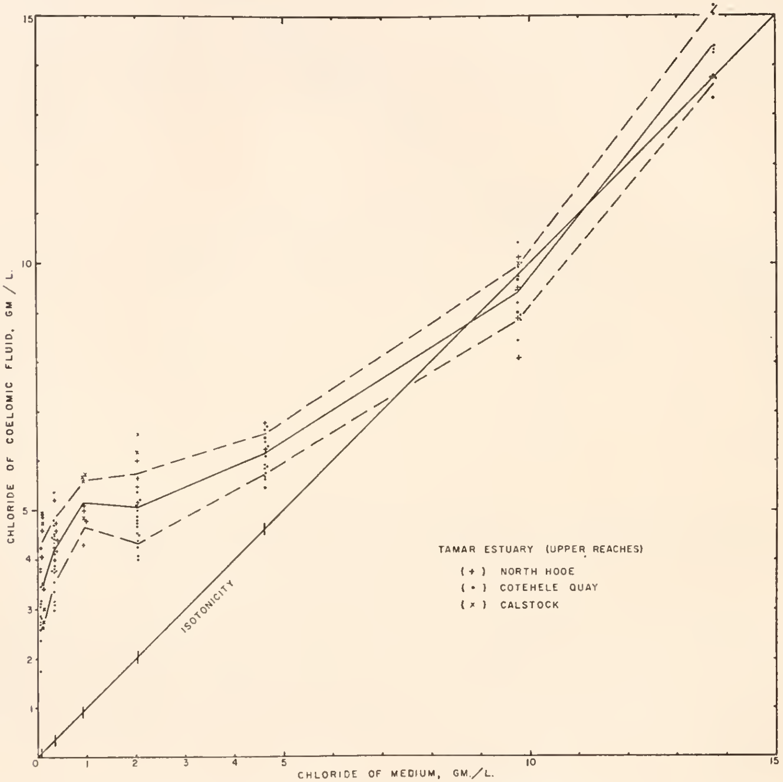

FIGURE 5. Chloride regulation in *N. diversicolor;* combined data from three stations, as indicated, on upper reaches of Tamar Estuary.

Differences in experimental technique likewise were unavoidable, but efforts were made to minimize them. One "break" occurred between the work at

Glasgow-Millport and the spring studies at Plymouth, since all reagents and standard solutions were made up afresh at the latter laboratory. However, a number of Millport worms had been brought to Plymouth and were tested after adaptation to Plymouth sea and tap water mixtures. The results (Fig. 1) suggest no discontinuity as a result of the change of scene. A second major break occurred between the spring work at Plymouth and that in Finland since (a colorimeter being unavailable) it was necessary to revert from Gordon's Fast Green modification back to an essentially unmodified Conway titrimetric method for chloride determinations. This change was prepared for by making up at Plymouth all the reagents to be used in Finland and transporting them there, together with all essential glassware. A single stock of NaCl standard was made up and divided into three lots, one used at Plymouth in the spring, one in Finland, and the third in Denmark. NaCl standard for the autumn work at Plymouth was made up in Denmark and checked against the previous lot before that was exhausted. The titrimetric Conway method proved satisfactory and was continued at Plymouth in the autumn in order to avoid a further change.

The greatest uncontrolled variable was undoubtedly temperature. This was in a sense uncontrollable, since the yearly temperature characteristics of each locality might be considered to have conditioned each population, so that bringing all animals to the same temperature would not constitute an equal amount of change from the environment to which each had become adapted. Accordingly, animals were simply tested at room temperature at each laboratory; this ranged from 12–18° C. in British laboratories in the cooler seasons to 18–24° C. in Finland and Denmark in summer. If it could be shown that such temperature differences could produce a significant effect upon the level of chloride regulation, the apparent similarity of results in the several localities might be an artifact masking actual differences between populations. The general problem of chloride regulation of aquatic animals in relation to temperature has recently been discussed in detail by Wikgren (1953), although data on polychaetes are lacking. On the basis of Wikgren's results on other animals it seems probable that adaptation would probably eliminate differences in level of regulation and that shock effects can be ruled out. Temperatures below 3° C., which Wikgren found to have marked effects on osmoregulation, were not approached in this work, but in order to have some assurance that temperature differences of the order of magnitude encountered in the present studies might not have shifted the level of chloride regulation, a brief comparative test was made of worms from Cotehele Quay, River Tamar, in late autumn, 1954. Groups were adapted at temperatures of 7°, 14°, and 20–21° C. for a week or more before sampling. The results of the temperature comparison are given below in Table VI and plotted in Figure 6; no significant differences in level of chloride regulation can be demonstrated, especially in the critical lower salinities.

The results of the above temperature experiment indicate that within the range of temperatures encountered in the present studies it is unlikely that the results could have been seriously influenced. However, the problem deserves further study, especially in relation to the inner Baltic population of *N. diversicolor,* which may be exposed for some weeks each spring to low salinities at temperatures close to zero.

Another variable is that of size of experimental animals. Worms between 150 and 250 mg. in weight have been preferred for convenience in handling, but at times

TABLE VI

Effect of temperature; Cotehele Quay population

Temp.	Chloride of media (range)	Number of worms tested	Mean cl. of body fluid	Std. dev. of body fluid cl.
7° C.	0.02 gm./l.	8	2.66 gm./l.	0.32
7° C.	0.42–0.43	8	3.92	0.57
7° C.	2.05–2.08	9	4.16	0.52
7° C.	5.18–5.38	8	5.12	0.85
14° C.	0.01–0.02	14	3.03	0.49
14° C.	0.36–0.39	20	4.03	0.58
14° C.	1.98–2.57	14	4.68	0.40
14° C.	4.70–5.13	13	6.06	0.37
14° C.	9.10–9.75	11	9.43	0.33
14° C.	13.36–14.08	10	14.36	0.71
21° C.	0.39–0.42	10	3.47	0.48
21° C.	1.71–1.75	8	4.08	0.36
21° C.	4.52–4.57	6	5.98	0.57

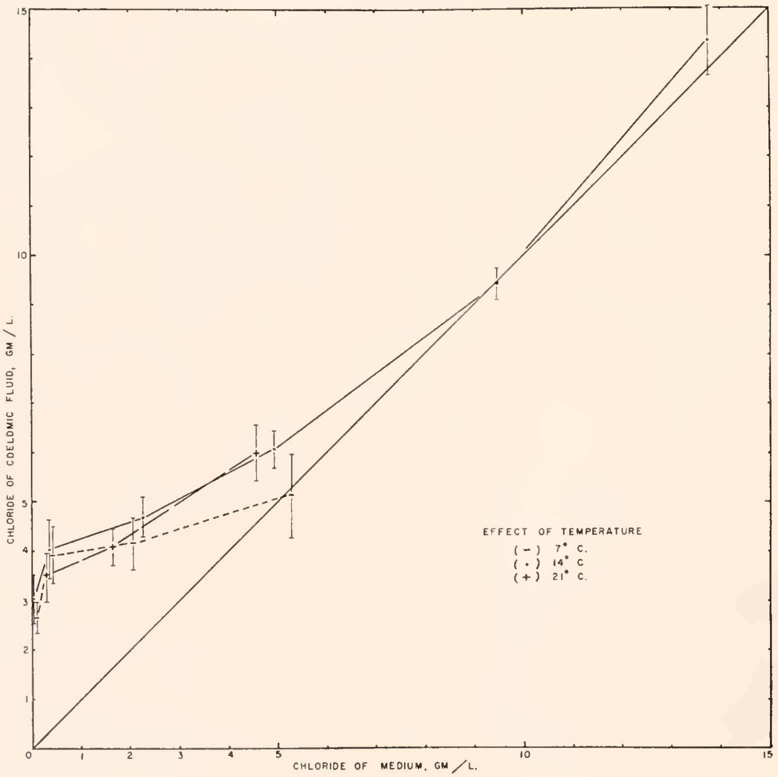

FIGURE 6. Chloride regulation curves for *N. diversicolor* from Cotehele Quay, upper River Tamar, obtained at temperatures of 7°, 14°, and 21° C. Vertical bars show ± one std. dev.

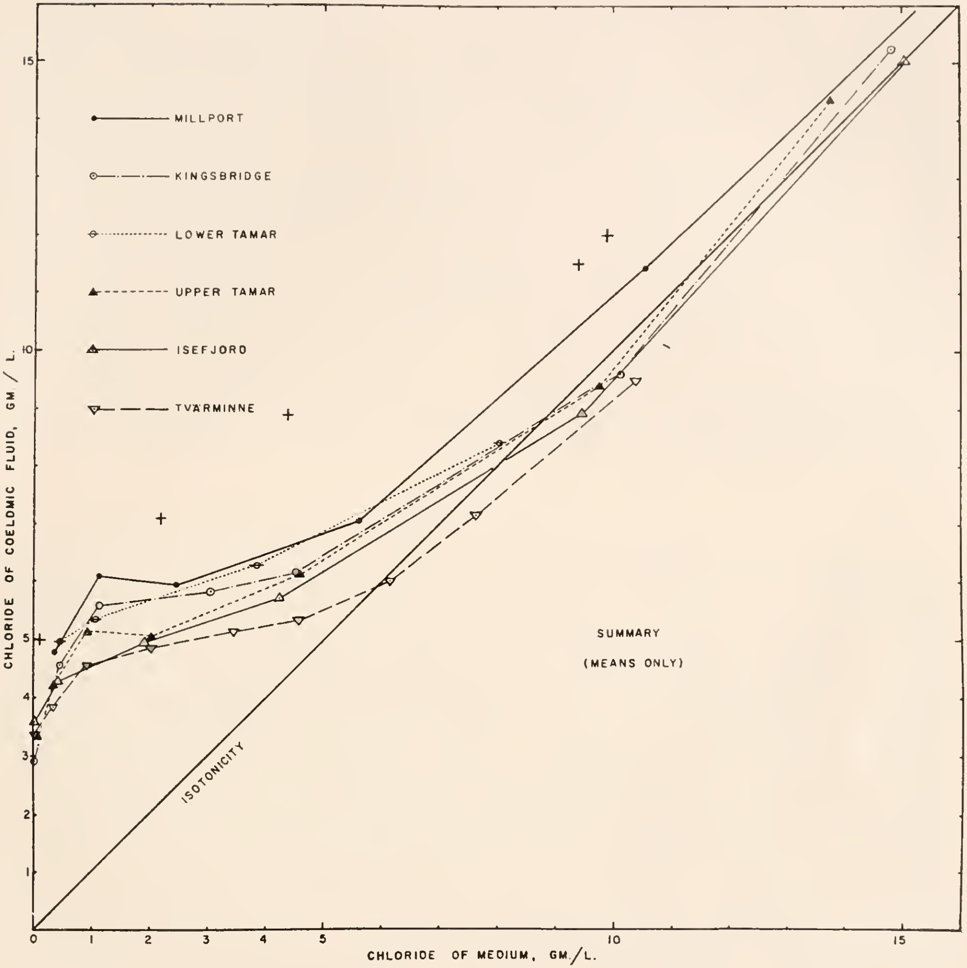

Figure 7. Chloride regulation curves (means only) from six localities, as indicated, shown together for comparison. Millport curve is for spring tests only; that for autumn tests would lie close to Tvärminne curve. Crosses show values estimated from data of Schlieper (1929) for *N. diversicolor* from Kiel region. Since Schlieper's data are freezing point depressions of press-juice, my estimation of chloride values is probably higher than would have been obtained on cell-free coelomic fluid, but the pattern of regulation is clear.

it has been necessary to use larger or smaller individuals. No relationship between body weight and level of chloride regulation has been noted, although on several occasions groups of large and small individuals have been compared in the same series of adaptations. This does not, of course, exclude the possibility that very small post-larval worms may, because of their high surface-volume ratio, encounter particularly severe osmotic problems; larval salinity tolerance is an aspect of the problem of physiological races in *N. diversicolor* which deserves study.

Finally, we have to consider whether or not experimental conditions might have depressed the level of chloride regulation in certain experimental series. That such is possible is suggested by the fact, mentioned earlier, that Millport worms adapted to dilutions of sea water made with Glasgow tap water showed a lower level of regulation than others from the same locality adapted to media made up with Loch Lomond water. A further occurrence, the cause of which is not clear, is illustrated in Figure 3, where one group of points may be seen to lie well below the general curve. The worms yielding these low values were retained for two weeks, care being taken that they were well fed and otherwise under good conditions, after which a second series of samples showed these worms to be "in line" with the rest of their series. In general, experimental animals were fed as much as they seemed to need; in one instance a group was left unfed for two weeks before testing, but without any effect being noted (Figure 1). There is no proof that all groups of worms were in equally good physiological condition. It can only be claimed that the results, except as just mentioned, have been obtained from worms which were apparently normal, which had been able to feed and to burrow, and which were able to re-burrow after the operation of sampling.

Discussion

In Figure 7 are plotted the curves of chloride regulation in the several populations sampled. The similarities between these curves are more striking than the differences. At first glance it might appear that worms from the more marine habitats showed a slightly higher level of chloride regulation than those from less saline waters, but no significance can be attached to such a conclusion. The reason for this is that *N. diversicolor* from marine-dominated habitats have been consistently less hardy under laboratory conditions than worms from low or variable salinities. It is difficult to assign a precise value to laboratory mortality, since deaths occurred over a considerable span of time, but anywhere from ⅓ to ½ of worms from Kingsbridge and Millport died in the laboratory in various experimental groups, and the curves represent results obtained from the survivors. If we make the reasonable assumption that the worms eliminated by death were those with the least regulatory ability, then the higher position of the curves for marine-dominated populations is an artifact. An ordinary statistical test for significance of the difference in level would be misleading, since the data are not drawn uniformly from the populations tested. On the other hand, laboratory mortality among worms from Tvärminne, the Isefjord, and the upper reaches of the Tamar Estuary was negligible, so that the curves for these groups represent essentially complete populations and are comparable among themselves. We conclude that under conditions of slow or stepwise adaptation, with feeding, at room temperature, *Nereis diversicolor* shows a constant level of coelomic chloride regulation relative to the salinity of the medium, regardless of the salinity of its regional background. This finding disposes of two suppositions regarding *N. diversicolor* in the Baltic Sea: it has not developed the ability to regulate at a higher level than its relatives elsewhere, nor has it significantly reduced its regulation in favor of adaptation of its tissues to low body fluid salinity (*note:* these experiments have not tested whether the tissues themselves might withstand lower salt concentrations; it is conceivable that they might be able to do so, and such an adaptation could be of survival value in Baltic worms exposed to the cold period of low salinity in spring).

Although in terms of the response measured in this study (level of coelomic chloride after careful adaptation) *N. diversicolor* is not separable into physiological races, the possibility of the existence of such races definable on other criteria has not been ruled out. In this discussion a physiological race has been thought of as a population (perhaps a section of a cline) genetically distinct in respect to some adaptive physiological response or capability. This definition of a physiological race requires that we recognize the environmental factors which have acted selectively upon the population, and that we devise appropriate criteria and tests for measuring the response to the factor in question under conditions which rule out the immediate effect of the environmental background. Although the criterion upon which the present study was based is apparently not diagnostic of physiological races in *N. diversicolor*, other lines of approach may prove more fruitful. For instance, there has been observed in this study a consistently higher mortality, or lower adaptability to salinity change, in certain populations from marine-dominated habitats. To what extent this represents a characteristically lower ability to withstand the emergency of salinity variation *per se* is not yet clear, since such worms have been ontogenetically conditioned to high salinity and have been forced in these experiments to undergo a far greater change of internal salt concentration than have worms from lower salinities. It must be recalled that chloride regulation does not take place at salinities much above 25–30 per cent of sea water, and that a drop in external salinity from 25‰ to 15‰ results in a greater change of tissue fluid salinity than does a drop from 15‰ to 5‰, in the range where regulation is effective. Another difference between populations which deserves further investigation is the failure (perhaps the result of my own inexperience) to adapt Millport worms to fresh water, although at least some of the marine-dominated Kingsbridge population would survive such exposure for at least two weeks, and essentially all Tvärminne, Isefjord, and upper River Tamar worms could do so for an indefinite period. A more critical study is needed before we can be certain that the Millport population is physiologically distinct in its response to minimal salinity. At present it cannot be said whether these worms were killed by the effect upon the tissues resulting from the magnitude of the salinity reduction which they experienced in spite of regulation, or whether death resulted from an actual breakdown of regulation at a certain lower limit of salinity. It is possible that under severe osmoregulatory stress certain ions may not be regulated properly, with the result that ionic imbalance in the body fluid may prove fatal, even though regulation of the total osmotic pressure may be adequate.

The adaptive responses of *N. diversicolor* to lowering of the environmental salinity are undoubtedly of several sorts, some of which may be susceptible of evolutionary modification in local races while others may be much more stable in the species as a whole. Among such protective physiological responses and mechanisms we may note the suggestion of Beadle (1937) that the ability of the body wall to resist osmotic distension is a primary protective device. This may well be so when salinity drops suddenly from a high value, for excessive swelling renders a nereid unable to swim, burrow, or to irrigate its tube, and the prevention of swelling of the body until other mechanisms come into play to relieve the stress may be of great survival value. Secondly, there is the ability to regulate the body volume after swelling by loss of salts and/or by production of isotonic urine, and so to reduce hydrostatic stress, as emphasized by Beadle (1937) and Ellis (1937).

Thirdly, there is the physiological task of the tissues in accommodating to the shock of falling salt concentration of the body fluids as the worm adjusts to salinity lowering above the regulatory level, a matter discussed by Wells and Ledingham (1940), who also pointed out that the tissues of different species, and possibly of local races, of polychaetes may differ in their ability to tolerate low salinities. Fourthly, there is the ability to regulate the internal osmotic pressure and salt concentration, to hold them relatively constant as the salinity of the medium falls to critically low values, as demonstrated by Schlieper (1929) and in the present paper. Then there is the further question of the response to fresh water, in which there is a possibility that an actual lowering of the permeability of the body wall may take place as a barrier against osmotic disaster, suggested in recent work of Jørgensen and Dales (in press). The role of the nephridia in osmotic and ionic regulation in brackish-water polychaetes, although a reasonable possibility, has never been directly demonstrated; the relatively greater size of the nephridia in brackish- and fresh-water nereids (Krishnan, 1952) suggests that they may be actively concerned. Beadle (1937) and Ellis (1937) assign the nephridia a role in elimination of excess water after osmotic swelling (possibly by means of hypotonic urine), but no studies of urine concentration in any polychaete have as yet been made.

The postulation of physiological races within the species *N. diversicolor* requires the recognition of environmental factors which have exerted selective pressure, an understanding of the pattern of variation of these factors in the range of the species, and the application of tests to measure the response to the factor in question under conditions which minimize the immediate effect of the local ecological background. If such criteria are employed, fewer physiological races may be postulated, but certain phenomena of distribution may be more readily explained as the result of particular environmental forces than by the assumption of physiological races. The criterion of level of body fluid chloride after slow adaptation has been shown in the present study to be non-diagnostic of physiological races in *N. diversicolor*. It seems quite probable, however, that some other type of response, either the response to fresh water or some form of emergency response to changes in salinity, may be diagnostic in this respect.

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Rasmussen of the Kgl. Veterinaer- og Landsbohøjskole, Copenhagen, for placing at my disposal his laboratory at Vellerup Vig, and for his generous advice and assistance in collecting.

SUMMARY

1. Laboratory studies have been made of the level of chloride regulation in *Neresis diversicolor* from representative parts of its geographical and ecological range. Areas studied include marine-dominated habitats in Scotland and southern England, estuarine habitats in southern England, conditions of low salinity in the Gulf of Finland, and intermediate salinities in the Isefjord, Denmark.

2. Animals have been adapted from their environmental salinity to a series of salinities from full sea water or higher down to fresh water, and the chloride level of the coelomic fluid has been determined after full adaptation and feeding at each salinity.

3. Results indicate that *N. diversicolor* as a species shows a uniform pattern and level of chloride regulation regardless of the region in which it is found. The test does not demonstrate physiological races differing in respect to salinity tolerance, although the possibility that other criteria may prove diagnostic of such races is not ruled out.

4. It is suggested that the assumption of the existence of physiological races in *N. diversicolor* should only be made after analysis of the environmental factors in question, and that certain differences of response and distribution may be caused by environmental rather than racial factors. For instance, the failure of *N. diversicolor* to populate oligohaline waters in Finland seems to be the result of local seasonal hydrographic factors rather than to indicate a physiologically distinct race of the species in that area.

Addendum: While this paper was in press there appeared a review by C. L. Prosser (1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262), in which there is an illuminating discussion of the nature of physiological races and the criteria necessary for their recognition. In the light of this review, special attention ought in future to be directed to the lower end of the chloride regulation curve of *N. diversicolor,* where the level of regulation drops or where the mechanisms of homeostasis fail in very low salinity or fresh water. It is clear that *N. diversicolor* behaves as a typical adjustor in higher salinities but as a regulator in lower salinities.

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THE KILLIFISH, FUNDULUS HETEROCLITUS, SECOND INTERMEDIATE HOST OF THE TREMATODE, ASCOCOTYLE (PHAGICOLA) DIMINUTA

HORACE W. STUNKARD AND JOSEPH R. UZMANN[1]

New York University and the U. S. Fish and Wildlife Service

Ascocotyle (*Phagicola*) *diminuta* was described by Stunkard and Haviland (1924) from the intestine of wild rats collected at the Clason Point dump near New York by the City Board of Health. Feeding experiments have demonstrated that metacercariae encysted in the gills of the common killifish, *Fundulus heteroclitus*, are stages in the life-cycle of this parasite. The larvae have been found also, although rarely and in small numbers, in the gills of the striped killifish, *Fundulus majalis*. Since both species of *Fundulus* are susceptible, it appears probable that the habits of *F. heteroclitus* occasion greater exposure of this species to the cercariae. It is possible that infection of the fishes occurs during the spawning season when they are in water of low salinity. The asexual stages of these trematodes and the mollusks which harbor them are yet unknown.

When Stunkard and Haviland described *A. diminuta*, they divided the genus *Ascocotyle* Looss, 1899 into two subgenera, *Ascocotyle* and *Parascocotyle*, with *A. coleostoma* (Looss, 1896) type of the genus as type of the subgenus *Ascocotyle* and *A. minuta* Looss, 1899 as type of the subgenus *Parascocotyle*. The two species appeared to differ significantly but the subsequent discovery and description of a number of additional species have disclosed intermediate conditions that seem to bridge the differences between the subgenera. Previously Faust (1920) had described worms from the intestine of the monkey-eating eagle of the Philippine Islands, *Pithecophaga jefferyi*, as members of a new genus and species, *Phagicola pithecophagicola*. According to Faust, "A close study of *Phagicola pithecophagicola* shows it to belong to a group possessing in part the characters of the Brachycoelinae, in part those of the Microphallinae, in part those that are unique." Accordingly, a new subfamily, Phagicolinae, was erected to receive it. The diagnosis of the subfamily was of course merely a list of the characters of the genus *Phagicola*. Actually the description of the worms was very incomplete and in a subsequent publication, Faust and Nishigori (1926) there is the statement, "In 1920 one of us (Faust) described a new species from the intestine of the monkey-eating eagle under the name *Phagicola pithecophagicola*, a fluke which on restudy has been found to belong to the genus *Ascocotyle* and should, therefore, be designated as *Ascocotyle pithecophagicola*." Witenberg (1929) raised *Parascocotyle* to generic status and transferred several species from *Ascocotyle* to *Parascocotyle*; among them *P. pithecophagicola* Faust, 1920. Although he recognized *Parascocotyle* as a valid genus, Witenberg considered *P. diminuta* specifically identical with *P. mi-*

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[1] Now located at the Western Fish Disease Laboratory, Fisheries Center, University of Washington, Seattle 5, Wash.

nuta (Looss, 1899) from dogs, cats and *Ardea cinerea* of Egypt. The genus was included in the subfamily, Centrocestinae, of the family Heterophyidae. Examination of original specimens of *P. pithecophagicola* by Witenberg did not yield further information and he (1930) reported that redescription from more favorable material would be required to determine whether or not *Parascocotyle* is identical with *Phagicola*.

The status of *Phagicola* is still unsettled. Travassos (1930) adopted the opinion of Stunkard and Haviland and recognized the two subgenera in *Ascocotyle*. However, he suppressed *Parascocotyle* as a synonym of *Phagicola*, included all previously described species in the genus *Ascocotyle* and arranged them in the two subgenera, *Ascocotyle* and *Phagicola*. The species of Faust was listed as *Ascocotyle* (*Phagicola*) *pithecophagicola*. *Ascocotyle* (*Phagicola*) *diminuta* was recognized as a valid species. Price (1932b) reported briefly on a re-examination of the type specimens of *Phagicola pithecophagicola*. He found the postoral appendage, which extended to the pharynx, two gonotyls in the genital sinus, and a globular seminal receptacle median to the ovary. With the description of these features, the first two of which were overlooked in the original description, Price announced the identity of *Phagicola* and *Parascocotyle*. He considered the differences between *Ascocotyle* and *Phagicola* to be of generic significance. Price (1932c) described two new species of *Ascocotyle* from the intestine of *Butorides* sp. taken in Puerto Rico, and reported finding *Phagicola diminuta* in the same host. Price (1933a) studied the original specimens and revised the description of *Ascocotyle plana* Linton, 1928 a species, taken from *Butorides virescens* at Woods Hole, Massachusetts, which had been regarded by Witenberg (1929) as identical with *Pygidiopsis genata* Looss and by Travassos (1930) as identical with *Ascocotyle* (*Phagicola*) *angrense* Travassos, 1916 from *Butorides striata*, *Butorides virescens*, and *Butorides erythromelas* of South America. The specimens were referred to the genus *Pygidiopsis* as *P. plana* (Linton, 1928). Price (1933b) published new host records for trematodes of the genus *Phagicola*. *Phagicola nana* (Ransom, 1920), originally described as *Ascocotyle nana* from the Alaskan fox, *Vulpes lagopus*, was reported from a booby bird, *Sula bassana*. In this species Price noted that the oral coronet consists of "a complete anterior row of 16 spines and an incomplete posterio-dorsal row of 3 to 4 spines." The specimens of *P. longa* (Ransom, 1920) from *Vulpes lagopus* were compared with Witenberg's description of this species from the dog, cat, and Persian wolf. According to Price, Ransom's material showed many abnormalities and the specific description was emended; Price reported *P. longa* also from an undetermined species of pelican. Ciurea (1933) described heterophyid parasites from mammals and birds, obtained by feeding fishes from the Danube and the Black Sea. After feeding metacercariae from the gills and superficial muscles of *Mugil capito* from the Black Sea to the dog and common cormorant, *Phalacrocorax carbo*, he obtained specimens presumably identical with those identified as *Parascocotyle longa* (Ransom, 1920) by Witenberg (1929). He noted that in these worms the acetabulum is located within the genital sinus and accordingly they were described as *Metascocotyle witenbergi* n. g., n. sp. Price (1935) described *Ascocotyle tenuicollis* n. sp. and published more extended descriptions and figures of *Ascocotyle megalocephala* Price, 1932, *Ascocotyle puertoricensis* Price, 1932 and of *Phagicola pithecophagicola* Faust, 1920. He stated that the location of the acetabulum within the genital sinus is common to all members of *Phagicola* and *Ascocotyle*, and accordingly the genus *Metascocotyle* Ciurea was suppressed as identical with *Phagicola*. Price disagreed with the opinion of Travassos (1930) that *Phagicola* should be considered a subgenus of *Ascocotyle*. As generic distinctions he stated, "Members of the genus *Ascocotyle* have 2 rows of spines in the oral coronet; the cuticula is entirely covered with spines; the uterus extends anterior to the genital aperture; and the vitellaria extend anterior to the level of the ovary. Members of the genus *Phagicola* have only a single row of spines in the oral coronet; the cuticular spines are absent at the posterior end of the body; the uterus does not extend anterior to the genital aperture; and the vitellaria are confined to the post-ovarial region of the body." Srivastava (1935) reviewed earlier work on the genus *Ascocotyle* and described *Ascocotyle (Phagicola) intermedius* from the Indian fishing-eagle, *Haliaeetus leucoryphus*. The specific name was selected because in certain respects the worms were intermediate in structure between *Ascocotyle* and *Phagicola*. They have a double row of oral spines and the vitellaria extend to the level of the pharynx, but the uterus does not enter the preacetabular zone. Accordingly, Srivastava reduced *Phagicola* to the rank of a subgenus and revised the diagnosis of *Ascocotyle* to contain the species formerly included in *Phagicola*. Price (1936) described *Ascocotyle mcintoshi* n. sp. from the white ibis, *Guara alba*, taken at Cape Sable, Florida. Commenting on the action of Srivastava, he argued that *Phagicola* is a valid genus since, "the species comprising the *Ascocotyle-Phagicola* complex fall quite distinctly into 2 categories—one group, *Ascocotyle*, having 2 rows of spines in the oral coronet, body completely spined and vitellaria extending anterior to the level of the ovary, and the other group, *Phagicola*, having a single row of spines in the oral coronet, the body incompletely spined (spines absent on posterior portion of body) and vitellaria confined to the postovarial region. In view of the fact that in each of these groups there are at least 3 correlated characters the writer regards *Ascocotyle* and *Phagicola* as better established genera than some of the other genera of heterophyids, as well as many genera of other families, the validity of which rests largely upon a single character which in many cases is decidedly variable." It is to be noted that Price (1936) had dropped the extent of the uterus as a generic character and there is doubt concerning the significance of the number of rows of oral spines. He (1933b) had reported that in *Phagicola nana* there is "a complete anterior row of 16 spines and an incomplete posteriodorsal row of 3 to 4 spines." We have found that in *Ascocotyle (Phagicola) diminuta* there are two dorsal spines of a second row. It appears, therefore, that in these species a second row is represented by a few persistent spines. If these spines are actually members of a second incomplete row of smaller spines, and in species of *Ascocotyle* the spines of the second row are smaller than those in the anterior row, the distinction between *Ascocotyle* and *Phagicola* rests on the extent of body spination and of the vitellaria. Moreover, the figure of *Ascocotyle puertoricensis*, published by Price (1935), shows that the vitellaria extend only a very short distance anterior to the ovary and do not reach the level of the genital aperture. Decision on the taxonomic status of *Phagicola* should await more complete information, especially on the developmental stages of its members.

Nishigori (1924) reported the life-cycle of *Stamnosoma formosanum*, a species which Price (1932a) transferred to *Centrocestus* on suppression of the genus *Stamnosoma* Tanabe, 1922. Yamaguti (1938) described the life-history and developmental stages of *Centrocestus armatus* (Tanabe). The asexual stages occur in species of *Semisulcospira,* the metacercariae in the gills of the freshwater cyprinid, *Pseudorasbora parva,* and the adults in *Milvus migrans lineatus.* Price (1940) gave a synopsis of the superfamily Opisthorchioidea Vogel, 1934 and discussed the adult morphology as well as the life-cycles and cercarial types of twenty species for which information was then available. Four families were recognized in the superfamily; two of them, Opisthorchiidae Braun, 1901 and Heterophyidae Odhner, 1914, contain species which are parasitic chiefly in warm blooded hosts and the others, Acanthostomidae Poche, 1926 and Cryptogonimidae Ciurea, 1933, contain parasites which usually occur in cold blooded hosts. Diagnoses were given for families and subfamilies, with lists of genera included in each. *Ascocotyle* and *Phagicola* were placed in the subfamily Centrocestinae, family Heterophyidae. Price stated that all described cercariae are pleurolophocercous or parapleurolophocercous but noted unexpected differences in the excretory systems of the cercariae which are unlike in shape of bladder, pattern of excretory ducts, and flame-cell formulae. It should be pointed out that the cercaria of *Centrocestus armatus* differs from other pleurolophocercous larvae in the absence of a caudal fin. Cable and Hunninen (1942), after describing the developmental stages of *Siphodera vinaledwardsi,* a member of the Cryptogonimidae, listed features common to the Heterophyidae, Cryptogonimidae and Acanthostomidae. All of the heterophyid cercariae are similar and it is probable that the as yet unknown cercaria of *Ascocotyle* (*Phagicola*) *diminuta* will prove to be a member of this larval group.

The original description of *Ascocotyle* (*Phagicola*) *diminuta* was made from moribund specimens found in the washings of the intestines of two rats that had been kept for several days in refrigeration. As a result, the account lacked precision in certain respects, especially the number of oral spines, and measurements made on such material may not be typical of normal individuals. It is recognized that trematodes, moribund in the intestines of their hosts or in the fluid of dissection, tend to relax and absorb water. Discovery of the metacercariae and experimental infections of rats, mice, hamsters, gulls (*Larus argentatus*) and a night-heron (*Nycticorax nycticorax*) have provided an abundance of vigorous, active worms. Accordingly it is possible to make additions and emendations to the description of the species.

The metacercariae were discovered by Uzmann in the gills of *Fundulus heteroclitus* collected in areas near the laboratory of the U. S. Fish and Wildlife Service at Milford, Connecticut. Examination of encysted specimens and others released from their cysts disclosed the resemblance between these worms and those described by Stunkard and Haviland (1924) from the rat. Feeding gills of *F. heteroclitus* to rats and mice yielded adult worms identical with those described earlier from natural infections. Gills of *F. heteroclitus* collected from the Clason Point area near New York and from Chesapeake Bay near Annapolis, Maryland, showed cysts of the same parasite. The incidence and intensity of infection varied little in fishes from different locations. Eighty-eight to ninety-six per cent of the fishes taken near the laboratory at Milford, Conn., harbored metacercariae and total counts made on twenty-five fishes varied from 0 to 218, with an average of 56 cysts per fish. Although *F. heteroclitus* bore heavy infections, examination of twenty-five *Fundulus majalis* from the Milford region showed only five of them infected and the number of cysts varied from one to eleven.

Cysts in the gills of *Fundulus* (Fig. 1) are oval and measure from 0.07 to 0.14 mm. in width and from 0.13 to 0.22 mm. in length. The cyst wall is composed of a thin, flexible and very tough membranous envelop, surrounded by a connective tissue capsule. The worms grow in their cysts; in smaller cysts they may not entirely fill the space and are not folded, whereas in mature cysts (Fig. 2) they are much larger and the body is doubled on itself. Excysted specimens, when extended, measure 0.116 to 0.33 mm. in length. In young cysts the larvae have small eye-spots, but in older ones the pigment is dispersed. The spines of the oral coronet increase in size as the larvae grow; they are visible in the cyst and provide a useful identifying character. The intestinal ceca of small metacercariae contain erythrocytes of *Fundulus*, and they may persist for some time. Metacercariae from fishes that had been kept in captivity for three months, during which time they had not been exposed to cercariae, had red blood cells in their digestive ceca. It appears that in penetrating the gills, the cercariae break the capillary beds and come to lie in small pools of blood. Blood must be ingested either before or after encystment and the red cells are not digested by the larvae. Eventually they decompose and disappear and they are not present in large metacercariae.

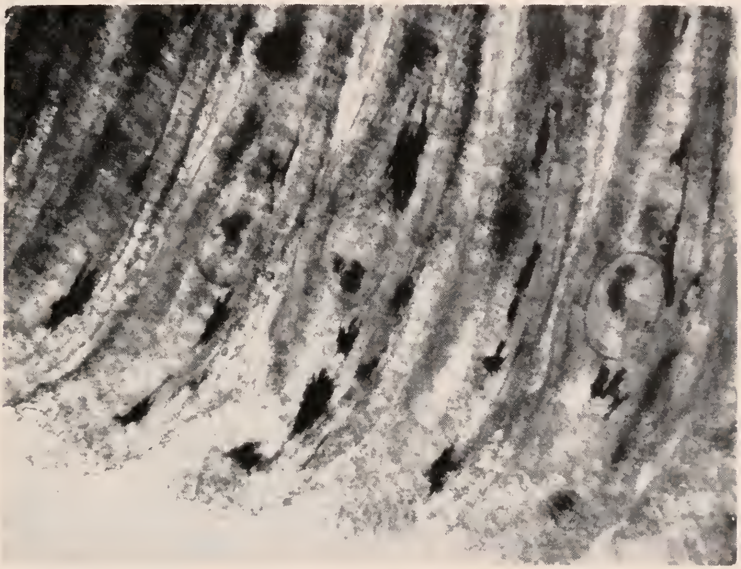

FIGURE 1. Photomicrograph, gills of *Fundulus heteroclitus*, showing encysted metacercariae of *Ascocotyle* (*Phagicola*) *diminuta*.

Gills were fed to rats, mice, hamsters, chicks, young laboratory raised gulls (*Larus argentatus*) and to a black-crowned night-heron (*Nycticorax nycticorax*).[2] All of these animals except the chicks, which proved refractory, were susceptible to infection and gravid worms were recovered in large numbers. The first rat, fed gill arches and filaments from five fishes each day for three days (Feb. 24, 25,

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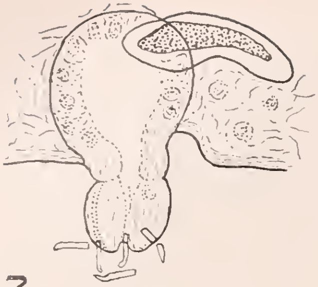
[2] This bird, a young female, was trapped in Bronx Park and provided through the cooperation of the New York Zoological Society.



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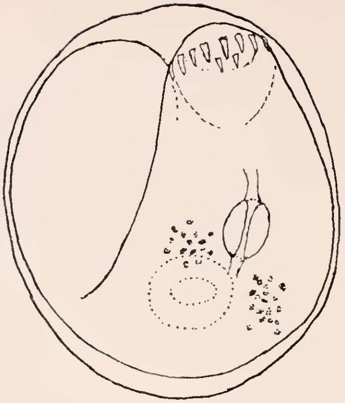

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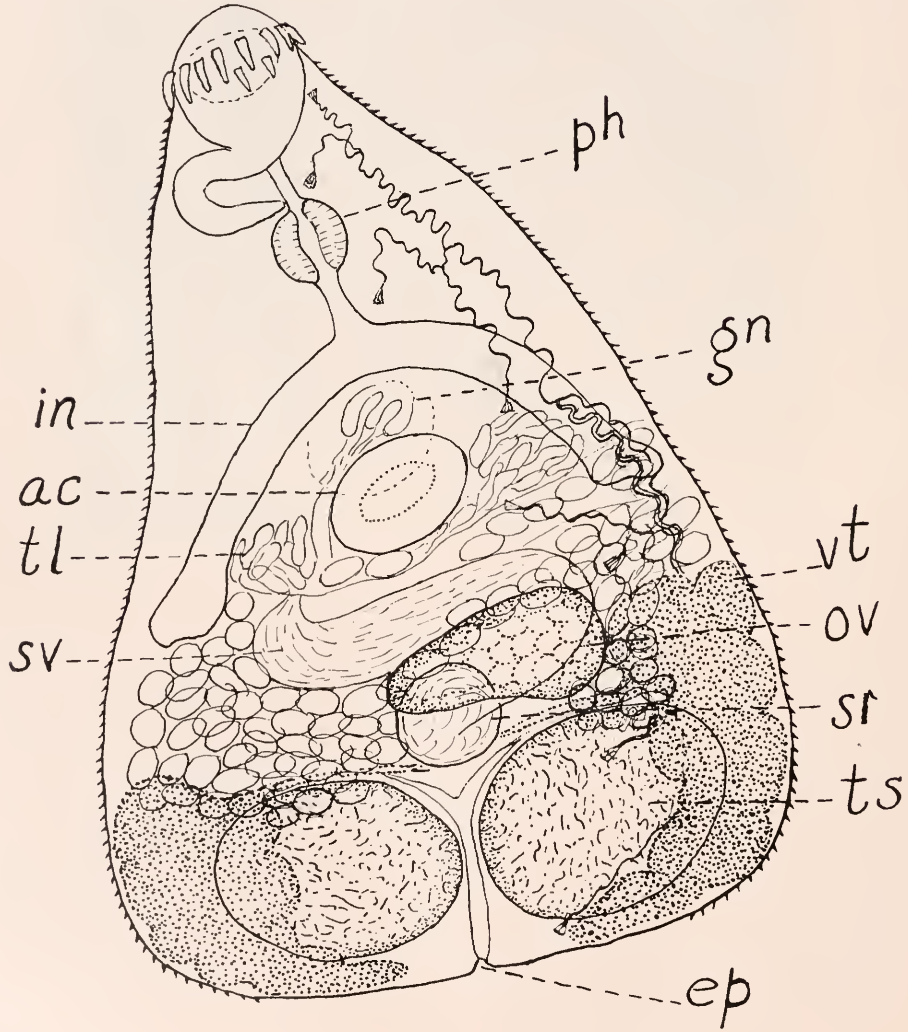
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Plate I

26, 1953) and killed four and one-half days after the first feeding, yielded over three hundred specimens, most of them sexually mature. Another rat, fed gills from four fishes on March 12 and from three fishes on March 14, was killed on March 23 and contained several hundred worms, all of them filled with eggs. A mouse killed two days after a single feeding contained almost one hundred worms, none of them with eggs. It appears that the worms may develop sexual maturity in three days, but worms from the smaller and younger cysts, although infective, require a longer time, as much as a week, to become mature, and they mature at a smaller size. Gills from two fishes were fed to a hamster and 253 gravid worms were recovered when the animal was sacrificed one week later. *Fundulus* were fed to laboratory-raised *Larus argentatus* which became infected. Also, six fishes were fed to a night-heron, *N. nycticorax*, and the bird was killed two days later. It contained about one hundred sexually mature worms of a natural infection and more than one hundred immature worms of the experimental feeding.

The worms recovered from the experimental infections of rats are more compact and robust than those from natural infections described by Stunkard and Haviland (1924). The specimen shown in Figure 4 is typical; it is one of the largest and as stained and mounted measures 0.31 mm. in length. In general, the worms are somewhat larger, with much larger gonads. Mature specimens vary from flask-shaped to triangular in outline, and the body is somewhat concave ventrally. The postacetabular portion, filled with reproductive organs and eggs, is much thicker than the more mobile anterior part of the body. The coronet of oral spines consists of a single row of sixteen spines and two additional spines, somewhat smaller than those of the complete row, situated on the dorsal side, immediately behind and on either side of the central spine. The flattened scale-like spines of the cuticula are absent from a narrow postoral zone or band and from the extreme posterior end of the body. The pigment from the eye-spots of the cercariae persists as dispersed granules in gravid worms. The degree of extension in the anterior part of the body determines the relative position of the oral appendage and the pharynx. When extended, the oral cecum may terminate some distance anterior to the pharynx as shown in the figure by Stunkard and Haviland, but when the body is contracted, as shown in Figure 4, the oral appendage may overlap the pharynx. The excretory system has been worked out in living worms and is depicted on the right side of Figure 4. The pore is terminal and the excretory vesicle is V-shaped with a short duct from the point of the V to the exterior. The two arms of the vesicle are dorsal and anterior to the testes and tend to follow their contour. When filled and compressed, the vesicle becomes reniform, overlying the

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FIGURE 2. Encysted metacercaria of *A. diminuta* from gills of *F. heteroclitus*, showing the coronet of oral spines, pharynx, acetabulum, and dispersed pigment of the larval eye-spots.

FIGURE 3. Portion of a transverse section of *A. diminuta*, showing everted gonotyl and spines. At the upper right of the figure there is a collapsed egg with contained miracidium.

FIGURE 4. Mature specimen of *A. diminuta* from the intestine of a rat, dorsal view, experimental infection, egg shells collapsed in the terminal loop of the uterus; abbreviations,

ac	acetabulum	sr	seminal receptacle
ep	excretory pore	sv	seminal vesicle
gn	gonotyl	tl	terminal loop of uterus
in	intestine	ts	testis
ov	ovary	vt	vitellaria
ph	pharynx		

testes with the ends of the vesicle extending almost to the lateral margins of the body, and anteriorly almost to the ends of the digestive ceca. On either side a collecting duct arises at the lateral end of the vesicle and extends forward in a sinuous course. A short distance anterior to the level of the acetabulum it divides into anterior and posterior branches, each of which bears two pairs of flame cells (Fig. 4). The arrangement of the flame cells on the posterior branch is the reverse of that on the anterior branch and the flame cell formula is $2[(2+2)+(2+2)]$. Counting backward from the anterior end, the first pair of flame cells is slightly ventral in position at the sides of the oral sucker; the second pair is dorsal in position anterior and lateral to the pharynx; the third pair is ventral, posterolateral to the pharynx; and the fourth pair is dorsal, preacetabular, above the digestive ceca. The fifth pair is ventral, lateral and slightly posterior to the acetabulum; the sixth pair is dorsal at the preovarial level; the seventh pair is dorsal, above the anterior edge of the testes; and the eighth pair is ventral, behind and below the testes. In the original report the relation between the acetabulum and the genital sinus was described but the gonotyl was not observed. In the more favorable material the gonotyl is recognizable but it assumes different shapes and is protrusible as shown in a transverse section (Fig. 3). The gonotyl bears very small spines, probably six in number, but they do not show well in whole mounts.

Other differences between present specimens and the original account are noted in the size of the gonads. It is clear that the specimens from which the original description was made were in very poor condition. In the experimentally produced worms the testes are large, the medial faces are in contact or even overlapping; the ovary is much larger, ovate in shape with the wider end lateral in position. The course of the uterus has been traced by following eggs as they passed along. As noted in the original description, the oviduct arises at the median posterior margin, the ootype is lined with long, rapidly beating cilia, the initial course of the uterus is posteriad on the right side of the seminal receptacle forming a loop along the anterior edge of the right testis, then forward to the right edge of the body, thence directly across to the opposite side where there is a posterior loop along the anterior face of the left testis, then across to the right side of the body where there is another loop and the uterus then crosses to the left side and forms another loop before the metraterm opens into the genital sinus. The eggs in the initial portion of the uterus have clear, transparent shells; as they pass along the shells become darker in color and those in the terminal loop are bright yellow. The eggs in the terminal coils of the uterus contain miracidia and eggs removed from gravid worms were kept under observation. Movement of the larvae was observed, but none of them emerged. Eggs were not recovered from the feces of infected rats, mice or hamsters, although gravid worms were found in the intestine of a rat killed six weeks after it was infected.

Measurements in millimeters of fixed, stained and mounted specimens are: length, 0.2–0.44; width, 0.11–0.24; diameter of oral sucker, 0.025–0.042; pharynx, 0.019–0.025; acetabulum, 0.03–0.04; testes, 0.03 by 0.04 to 0.05 by 0.07; ovary, 0.03 by 0.04 to 0.035 by 0.056; eggs, 0.016 by 0.010 to 0.02 by 0.011.

Sexual development of animals is dependent on nutritional and other physiological factors and its extent is often anomalous or irregular in parasitic worms from abnormal hosts. The variations in the degree of sexual maturity of *Ascocotyle diminuta* in mammals and the failure to recover eggs of the parasite in the feces of these hosts suggest that the natural hosts are piscivorous birds. Price reported the species from *Butorides* sp. and we have found it in *Nycticorax nycticorax*. These herons are common in the Milford area and feed in creeks where *Fundulus* is abundant.

SUMMARY

The metacercarial stage of *Ascocotyle* (*Phagicola*) *diminuta* Stunkard and Haviland, 1924 has been found encysted in the gills of *Fundulus heteroclitus*. Experimental infections of rats, mice, hamsters, *Larus argentatus* and *Nycticorax nycticorax* have yielded adult worms and permitted additions and emendations to the description of the parasite.

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THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. III. PHYSIOLOGICAL CHANGES WHICH OCCUR IN THE BLOOD AND URINE DURING THE NORMAL MOLTING CYCLE[1]

DOROTHY F. TRAVIS[2]

Bermuda Biological Station[3] and The Biological Laboratories, Harvard University and Radcliffe College, Cambridge 38, Mass.

In a previous paper (Travis, 1955) it has been shown that external manifestations of an approaching molt in the exoskeleton of the spiny lobster, *Panulirus argus* Latreille, reflect the more basic internal changes which occur in the integumental tissues and hepatopancreas. It might also be said that the internal body fluids, hemolymph and urine, to a large extent reflect physiologically those variations which occur both in the tissues and in the skeleton. Like those variations which occur in the tissues and exoskeleton, these changes in the body fluid are cyclical. Thus the cyclic periods of growth in the Crustacea are not only accompanied by periodic modifications in the exoskeleton, epidermis, sub-epidermal tissues, and hepatopancreas but are accompanied as well by marked alterations in the blood and urine. The present paper will therefore be concerned with the relation of certain alterations in the internal body fluids of *Panulirus* to those changes which occur in the exoskeleton and tissues.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

Stages of the molting cycle were designated by time intervals, in days, as previously described (Travis, 1954, 1955) and by the method of Drach (1939).

Determination of resorption in the area of softening

To determine the amount of resorption in the area of softening of *Panulirus,* chemical analyses were carried out on small pieces of exoskeleton. These pieces

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[2] Present Address: Department of Zoology, University of New Hampshire, Durham, New Hampshire.

[3] Contribution No. 222.

were taken from females 83.0 mm. in carapace length. Pieces of hard (late Stage C) and cast (Stage D) skeletons of nearly identical dimensions (12.8 mm. × 24.4 mm.) were used. The dry weights of these pieces were obtained and the per cent loss in weight of the piece from the shed skeleton calculated. The weight of ash was found and the weight of organic material of each piece was determined by subtraction. This was followed by an analysis of the ash for CaO, P_2O_5, and MgO in each piece. The per cent resorption of mineral and organic matter was then calculated.

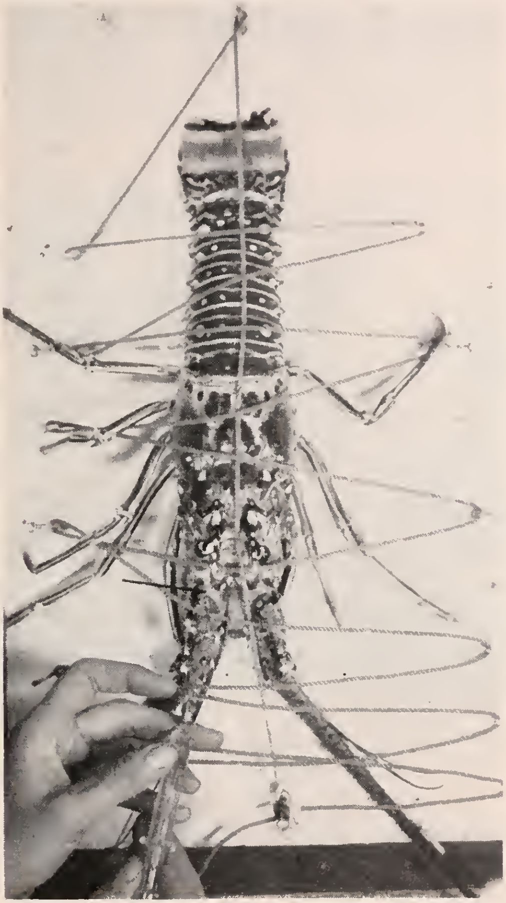

FIGURE 1. Method of securing blood samples from the antennal sinus (arrow) after animals were immobilized to an operating board.

Collection of blood and urine

For securing blood samples, it was necessary to fasten an animal to an operating board as is indicated in Figure 1. A needle of a hypodermic syringe was then inserted through the intersegmental membrane of the second joint of an antenna and blood was withdrawn from the large antennal sinus. The blood was placed in test tubes with anticoagulant added, and the cellular elements were allowed to aggregate with gentle shaking, after which the tubes were centrifuged and the plasma removed for analysis. It should be pointed out that the anticoagulant used was a natural one obtained from the animal's own blood and tissues. This was collected by splitting the animal dorso-ventrally down the midline and allowing the anticoagulant contained in the blackened blood to collect in a white enamel tray. The crude blackish material was centrifuged and the supernatant decanted from a gray precipitate. Three drops or 0.1 ml. of the black supernatant per ml. of blood was enough to prevent coagulation. This amount of the crude anticoagulant contained approximately 0.7 mg. of Ca. Appropriate corrections in the plasma calcium values were accordingly made. If kept in the refrigerator, such crude material is stable for as long as 6 months. Numanoi (1938) found a similar natural anticoagulant in the hepatopancreas of *Ligia*. No such substance could be obtained from the hepatopancreas of *Panulirus* either upon allowing the liver to stand or upon trituration. These natural anticoagulants of Crustacea apparently prevent the explosion of explosive cells, thus inhibiting the clotting process. The problems of the origin, properties, and identity of the anticoagulant are interesting ones for further investigations.

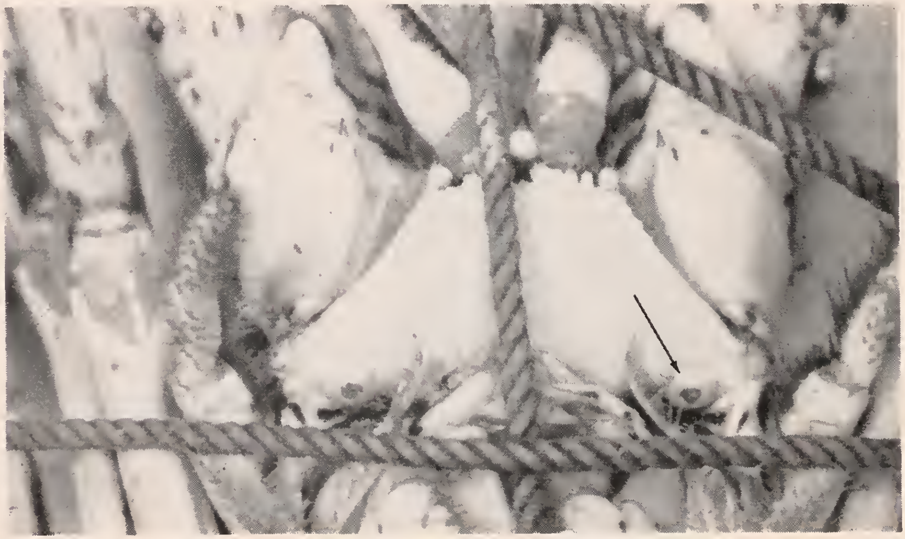

FIGURE 2. Urine was withdrawn from excretory openings, one of which is indicated by the arrow, after the animals were immobilized, dorsal side down, to an operating board.

For urine calcium analyses, the animal was secured to the operating board as indicated in Figure 2. Clear urine was withdrawn by inserting the rounded, smooth tip of the needle of a hypodermic syringe between the two flaps which guard the excretory opening to the bladder. It is only necessary to insert the syringe needle 1–2 mm. beyond the flaps to withdraw adequate quantities of urine for analysis.

Chemical analyses of blood and urine

The Clark-Collip (1925) modification of the Kramer-Tisdall (1921, 1923) method was used for blood and urine calcium determinations. Blood inorganic phosphate was determined on protein-free filtrates by the method of Fiske and SubbaRow (1925). Total blood phosphorus was determined by digestion of the blood samples with sulfuric acid and subsequent oxidation with 30% hydrogen peroxide. The phosphate-containing solution was then analyzed by the method of Fiske and SubbaRow. The values for calcium and phosphorus (both inorganic and total) are stated in terms of milliequivalents per liter so that their relative magnitudes are more effectively displayed. This value (mEq/L) is obtained by dividing milligrams per liter by the atomic weight and multiplying by the valence. With phosphorus compounds, the difficulty lies in the determination of the correct valence, thus making it difficult to obtain precise values. When phosphate is present in the body fluids, it exists either as $H_2PO_4^-$ and/or $HPO_4^=$, the relative percentage of these concentrations depending upon the pH of the body fluids, with H_2PO_4 having a valence of one and HPO_4 a valence of two. Unfortunately, the pH of *Panulirus* blood has not been determined throughout the entire molting cycle because of inherent difficulties in making accurate determination. However, Damboviceanu (1930, 1932) has shown that in *Astacus* the pH range is from 7.7–8.2 at various stages of the molting cycle. Numanoi (1937) similarly showed a range from 7.3–7.7 in *Ligia*, while Drilhon (1935) showed in *Maia* a pH range of 7.1–8.4. Baumberger and Olmstead (1928) showed that in *Pachygrapsus* the range was from 7.8 to 8.4. At these pH ranges reported for Crustacea most of the phosphate present in the body fluids would exist as a divalent radical (HPO_4^{--}); a much smaller percentage, however, would exist as a univalent radical ($H_2PO_4^-$). Although assuming a valence of two is not entirely correct, the author feels that in view of the paucity of information concerning crustacean blood, this assumption is perhaps no more incorrect than to assume a valence of slightly less than two, because of the fluctuations in pH during various stages of the molting cycle. For example, at a pH of 7.4 the valency of combining power of phosphorus would be 1.8 and not 2, at a pH of 7.8 the valency would be 1.9 and so on. Furthermore, the author would also like to point out that with regard to total phosphorus, the values are stated in terms of mEq/L, using 2 as the valence, which is not entirely correct because organic phosphorus in the total phosphorus value has no electronegative value. Values stated in millimoles per liter would be perhaps more appropriate but total phosphorus values are stated in terms of chemical equivalence so that the relative magnitudes of total calcium, inorganic and total phosphorus can be compared more easily.

Total blood protein was determined by the micro-Kjeldahl method which consisted of the Kjeldahl type digestion and oxidation (Hawk, Oser and Summerson, 1948). Appropriate correction was made for the non-protein nitrogen, which was determined in a separate analysis and subtracted from the total nitrogen value to give protein nitrogen. This value was then multiplied by a factor of 6.25 to convert grams of nitrogen to grams of protein. The factor of 6.25 is used for conversion because the average protein contains 16% nitrogen.

For each day on which analyses were made during the molting cycle, it was thought best to use as many different animals as possible. In most cases, not more than two to three blood samples per week were taken from any one animal. Never more than four ml. of blood were taken at any one time. This precaution was taken to prevent significant decreases in protein content of blood. In a few instances daily samples were taken from animals during a period of four days preceding molt (Stage D) to a period of fourteen days following molt (Stage A, B, and early Stage C). Though the trend was the same, the blood protein values were considerably lower than those values obtained in animals in which 2–3 blood samples per week were taken. It was therefore necessary to use as many different animals as possible at each stage of the molting cycle. Analyses in duplicate were carried out in most cases.

RESULTS

Resorption from the area of softening

Results from analyses of the area of softening (performed by Mr. A. F. Gonyer, Analytical Chemist, Department of Mineralogy, Harvard University) are recorded in Table I. It will be observed that a comparison of the dry weights of the two pieces of exoskeleton taken from the area of softening in a hard, late Stage C animal, and the shed skeleton (end of Stage D) shows that there is approximately 20% loss in weight. Therefore, the total resorption of organic and mineral material in this area is about 20%. Of the total organic material, including CO_2, 23% is resorbed and of the total mineral material approximately 13% is resorbed. The most abundant mineral element making up the exoskeleton is calcium, followed by magnesium and phosphorus (all determined as oxides). Of the total of each mineral element determined as the oxide, 8.4% calcium oxide, 20.0% magnesium oxide, 14.3% phosphorus pentoxide and 45.4% of minerals not identified are resorbed from the area of softening preceding molt.

From the information obtained, specifically from the area of softening, it is of interest to calculate the approximate amounts of various minerals and the organic material which would be resorbed from an entire carapace of a hard (late Stage C)

TABLE I

The amount of mineral and organic resorption in pieces of skeleton from the "area of softening" preceding molting

Material	W_1 Hard skeleton (late Stage C) weight in mg.	W_2 Shed skeleton (end of Stage D) weight in mg.	D Difference $W_1 - W_2$ (actual amount resorbed) mg.	Per cent loss in weight or % resorption (D as % of W_1)
Dry weight	155.9	125.2	30.7	19.7
Weight of dry ash (mineral)	51.1	44.6	6.5	12.8
% of the total dry weight as mineral	32.8	35.6		
Weight of organic including CO_2	104.8	80.6	24.2	23.0
% of the total dry weight as organic including CO_2	67.2	64.4		
Weight of total mineral ash as CaO	40.4	37.0	3.4	8.4
Weight of total mineral ash as MgO	3.5	2.8	0.7	20.0
Weight of total mineral ash as P_2O_5	2.8	2.4	0.4	14.3
Weight of total mineral ash unidentified	4.4	2.4	2.0	45.4

TABLE II

Calculated amounts (approximate) of mineral and organic material resorbed (preceding molt) from a carapace of 83 mm. length

Dry weight of 83 mm. carapace used (mg.)	Mineral and organic material	Amount of material (mg. per 100 mg. carapace)	Approximate amounts (mg.) of mineral and organic material in the carapace (calculated from columns 1 and 3)	Per cent resorption of mineral and organic material (Table I)	Approximate amounts (mg.) resorbed from the carapace preceding molt (calculated from columns 4 and 5)
41,500	CaO	25.9	10,749	8.4	903
	P_2O_5	1.8	787	14.3	112
	MgO	2.2	913	20.0	183
	Organic	67.2	27,888	23.0	6,414
	Mineral unidentified	2.8	1,162	45.4	528

animal (83 mm. carapace length). In this case the dry hard carapace was weighed without the endoskeleton and legs. The mandibles, two joints of the antenna, the eyes and three joints of the antennules were attached (see Figure 1, Travis, 1954). If one makes the assumption that the carapace contains the same proportion of mineral and organic material throughout, as was found in the area of softening, and that resorption occurs equally throughout the carapace, which is not the case, the approximate amounts in milligrams of mineral and organic material resorbed preceding molt can be calculated. These values are shown in Table II. All of these values are perhaps conservative figures since Drach (1939), and Drach and Lafon (1942), who have thoroughly studied resorption, have shown that unequal resorption occurs in the decapod skeleton. Complete resorption occurs in some parts of the endoskeleton while partial resorption occurs in most of the exoskeleton. The degrees of resorption in the exoskeleton are evident from

TABLE III

Feeding and starvation experiments

Analyses made	Treatment				Standard error of the difference	Probability values
	Fed daily 1 week		Starved for 1 week			
	No. used	Average value obtained mEq/L	No. used	Average value obtained mEq/L		
Total blood calcium	4	27.4±1.01	4	27.8±0.5	0.4 ±1.0	P=0.7–0.8
Blood inorganic phosphate	4	0.40±0.01	4	0.43±0.04	0.03±0.04	0.4–0.5
Urine calcium	4	36.4±4.6	4	37.8±0.5	1.4 ±5.0	0.7–0.8
	Fed daily 2 weeks		Starved for 2 weeks			
Total blood calcium	3	28.3±1.5	4	29.2±1.0	0.3 ±2.0	P=0.8–0.9
Blood inorganic phosphate	3	0.37±0.01	4	0.28±0.00	0.09±0.01	0.01
Urine calcium	3	33.4±2.6	4	32.4±2.0	1.0 ±4.0	0.8–0.9

gross observation and from histological studies (Travis, 1954, 1955). Without almost complete resorption in certain regions and at least partial in others, it would be impossible for the arthropod to expand the new soft body beneath the old skeleton preceding molt and to shed its skeleton at molt.

Effect of feeding and starvation on blood calcium, urine calcium and blood inorganic phosphate

Experiments were performed to determine whether or not food plays a significant role in influencing total blood and urine calcium, and blood inorganic phosphate levels. Two groups of animals were used. One group was fasted and the other was fed every day. These experiments covered a period of two weeks, and analyses were made at the end of the first and second weeks. The results of these experiments (Table III) indicate that starvation fails to affect significantly

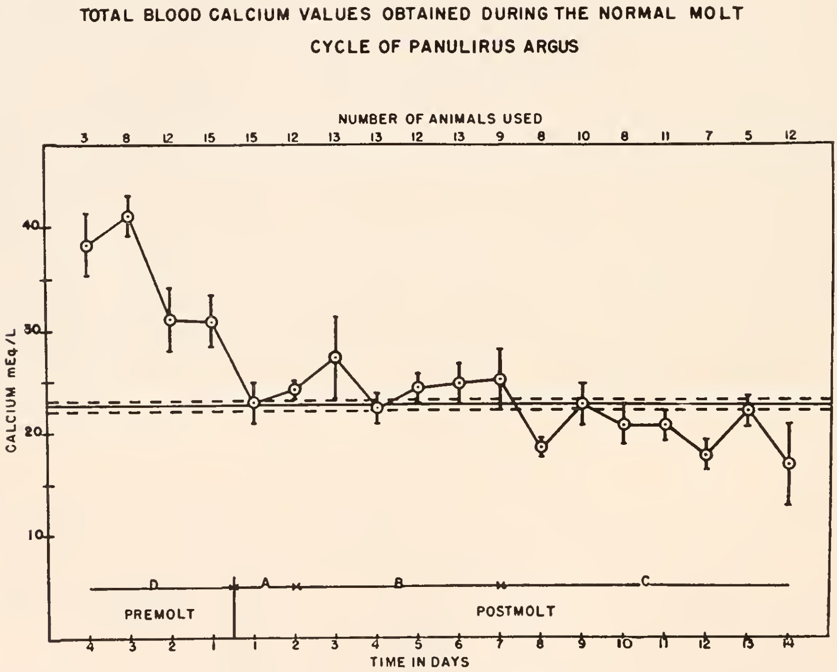

FIGURE 3. Changes in total blood calcium during the normal molting cycle. The number of animals used per point on the graph is indicated along the top ordinate. All points represent the mean arithmetical values obtained from duplicate analyses on this number of animals. The vertical lines indicate plus or minus the standard error of the mean calculated by Fisher's (1948) method for a small sample. The solid horizontal line indicates the mean values obtained from 80 intermolt (late Stage C) animals. Dashes above and below the solid horizontal line indicate plus or minus the standard error of the mean. The vertical line at the bottom of the graph separates the premolt and postmolt period. The bottom ordinate represents the time in days preceding and following molting. The solid horizontal lines parallel to the bottom ordinate and broken by the letters D, A, B, and C designate the four major stages in the molting cycle (Drach, 1939).

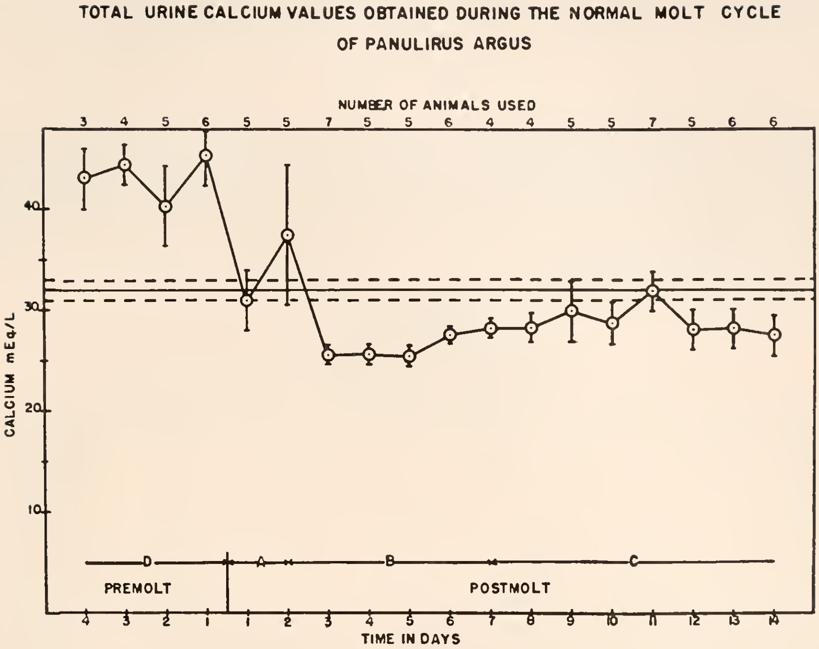

FIGURE 4. Changes in total urine calcium during the normal molting cycle. The solid horizontal line in this case indicates the mean arithmetical values obtained from 24 intermolt (late Stage C) animals.

blood or urine calcium levels. However, blood inorganic phosphate does appear to be altered by starvation. This shows up significantly by the end of the second week of such treatment.

Total blood calcium

It is apparent from the preceding results that a large amount of calcium is removed from the old skeleton before molt. As a consequence of this there is a marked rise in total blood calcium preceding molt (Fig. 3). The peak of this rise is reached three days before molt (late Stage D) at which time the resorptive or ecdysial line is clearly apparent. Therefore, appreciable resorption has already occurred in the area of softening and generally throughout the skeleton (Travis, 1954, 1955). This rise in blood calcium occurs three days before the increase in body weight occurs and before water uptake is evident. An increase of 18.4 mEq/L over the normal intermolt (late Stage C) blood levels is evident at the peak premolting value. During postmolt, Stages A, B, and early C, blood calcium levels in *Panulirus* remain approximately normal.

Total urine calcium

Urine calcium (Fig. 4), like blood calcium, is significantly higher than the normal intermolt (late Stage C) value preceding molt and then falls rapidly one day following molt (Stage A), reaching a subnormal value at 3 days (Stage B). The values approach normal levels around the ninth day (very early Stage C).

Urine calcium-blood calcium ratio

The ratio of urine calcium to blood calcium in normal intermolt animals (late Stage C) is 1.4. It will be observed from Table IV that there appears to be a significant departure from this ratio four and three days preceding molt (Stage D) and three through seven days following molt (Stage B), otherwise it remains relatively constant throughout.

Table IV

Urine calcium-blood calcium ratios obtained from Panulirus argus during the normal molting cycle

Stage of the molting cycle	Days before molt	Days after molt	$\frac{\text{Urine calcium}}{\text{Blood calcium}}$ Ratio
D	4		1:1
	3		1:1
	2		1:3
	1		1:5
A		1	1:3
		2	1:5
		3	0:9
B		4	1:1
		5	1:0
		6	1:1
		7	1:1
		8	1:5
		9	1:3
C		10	1:4
		11	1:5
		12	1:6
		13	1:3
		14	1:6
Late Stage C		28–35 (Normal Intermolt)	1:4

Total blood protein

It will be observed from Figure 5 that blood protein increases preceding the molt, declines following the molt and reaches a subnormal value by the third day (Stage B). Values remain below normal throughout most of the postmolt observation period (Stage A, B and early C).

Total blood phosphorus

Total blood phosphorus (Fig. 6) rises significantly above normal intermolt (late Stage C) values preceding the molt, but the fall to normal or subnormal values occurs earlier (two days preceding the molt, late Stage D) than was observed with blood calcium. Values remain below normal from the second through the fourteenth day following molt (Stage B and early Stage C).

Blood inorganic phosphate

No significant change occurs in inorganic phosphate preceding molt (Fig. 7). Following molt a significant fall occurs at seven days (end of Stage B) and blood levels remain below normal through the remaining fourteen-day period (early Stage C).

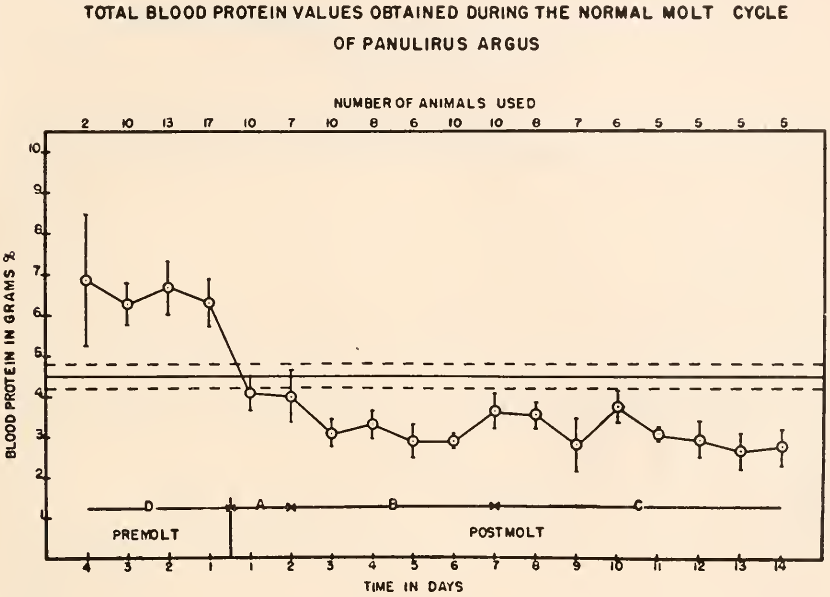

Figure 5. Changes in total blood protein during the normal molting cycle. The solid horizontal line indicates the mean arithmetical values obtained from 16 intermolt (late Stage C) animals.

Discussion

Before considering the blood and urine changes associated with the molting cycle, other pertinent aspects which have a direct bearing on the results obtained will be reviewed. The decapod Crustacea, enclosed in a heavily calcified exoskeleton, periodically shed their old skeleton as a necessary means of growth. Molt is preceded by resorption of considerable quantities of calcium, as well as other mineral and organic constituents. Complete mineral and organic resorption occurs in certain zones of the endophragmal skeleton while in most of the exoskeleton only partial resorption occurs (Robertson, 1937; Drach, 1939; Drach and Lafon, 1942; Travis, 1951a, 1954, 1955). Large amounts of these resorbed substances, therefore, must pass into the blood (Table II). The crustacean is then confronted with the problem of handling these excess constituents by either excretion or storage. Both methods may be resorted to. The method employed largely depends upon the availability and need of the constituents. For example, the phosphorus and some of the resorbed calcium are stored in the hepatopancreas of *Carcinus maenas* (Schönborn, 1912; Robertson, 1937), *Cancer pagurus* (Paul and Sharpe, 1916), *Maia squinado* (Drach, 1939), *Hemigrapsus nudis* (Kincaid and Scheer, 1952), and in *Panulirus argus* (Travis, 1955).

When the animal sheds its old skeleton, the new soft body increases in size by the absorption of water (Travis, 1954). One of the major tasks that the animal now encounters is that of hardening, by mineral deposition, its new skeleton. In some instances any available sources of the constituents necessary for hardening must be drawn upon to the full extent. In other instances this is not necessary because of the availability of the required element. Calcium, abundant in the Bermuda sea water (around 25–27 mEq/L), presents no problem to the spiny lobster. The problem of obtaining phosphorus is somewhat different. The small amounts of inorganic phosphate (about 0.04 mEq/L) available in Bermuda sea water are far less than those present in the blood. Sea water is neither the most important nor the most ready source of this element. In order to obtain it from sea water the spiny lobster must actively concentrate it. It is, therefore, necessary for this

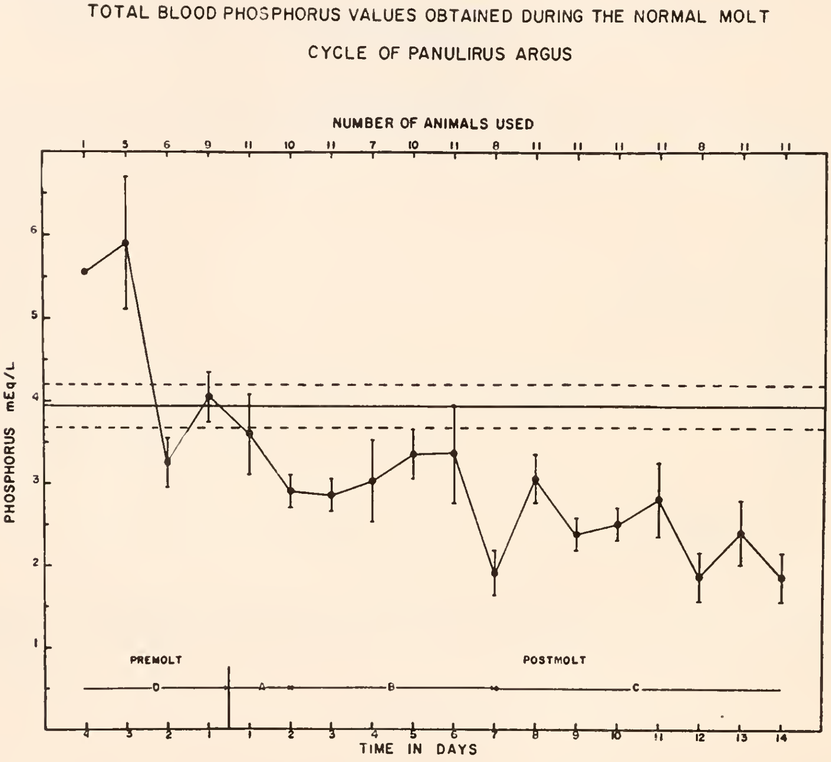

FIGURE 6. Changes in total blood phosphorus during the normal molting cycle. The solid horizontal line indicates the mean arithmetical values obtained from 11 intermolt (late Stage C) animals.

animal to conserve phosphorus. Furthermore, the feeding and starvation experiments (Table III) indicate that starvation significantly affects blood inorganic phosphate after two weeks of such treatment. The food, therefore, would appear to be the most important source of this element. Conservation of phosphorus in *Panulirus* must begin, therefore, long before shedding occurs because the animals undergo a period of inanition two weeks preceding and one week following molt (Travis, 1951a, 1951b, 1954, 1955). With a consideration of the foregoing, the blood and urine changes associated with the molting cycle will be discussed. These physiological changes observed in *Panulirus* are summarized in Figure 8.

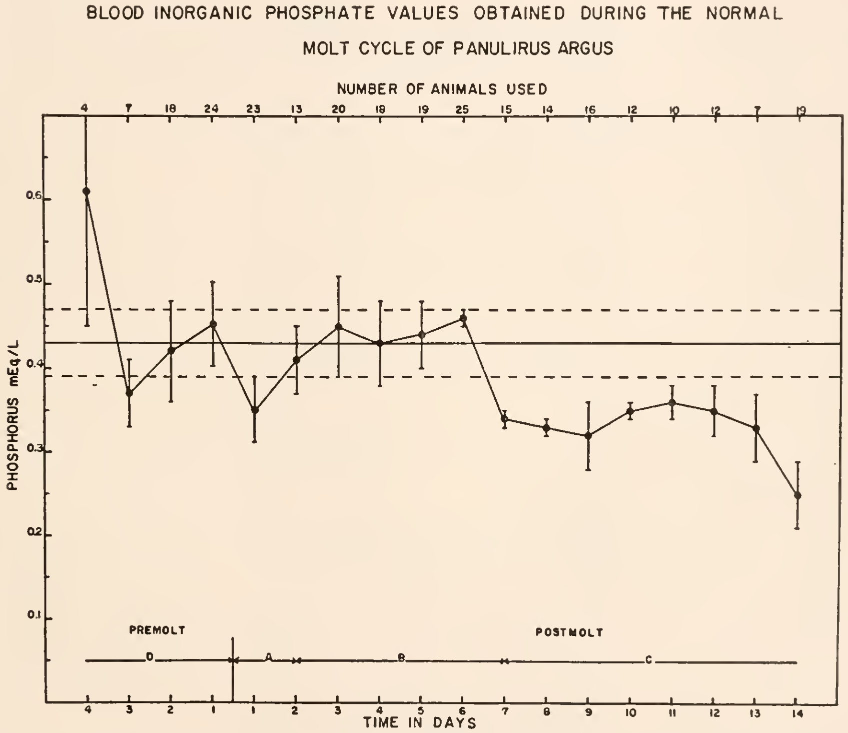

FIGURE 7. Changes in blood inorganic phosphate during the normal molting cycle. The solid horizontal line indicates the mean arithmetical values obtained from 115 intermolt (late Stage C) animals.

Total blood calcium

Total blood calcium in *Panulirus* almost doubles in concentration during the premolt period (late Stage D). This is the time at which the resorptive or ecdysial line and area of softening become apparent along the lateral portions of the carapace. Since mineral resorption is generalized and occurs throughout the skeleton, large amounts of calcium would appear in the blood at this time. The approximate amounts (Table II) resorbed at molt from a carapace (83 mm. in length) would suggest that the resorbed calcium from the old skeleton could ac-

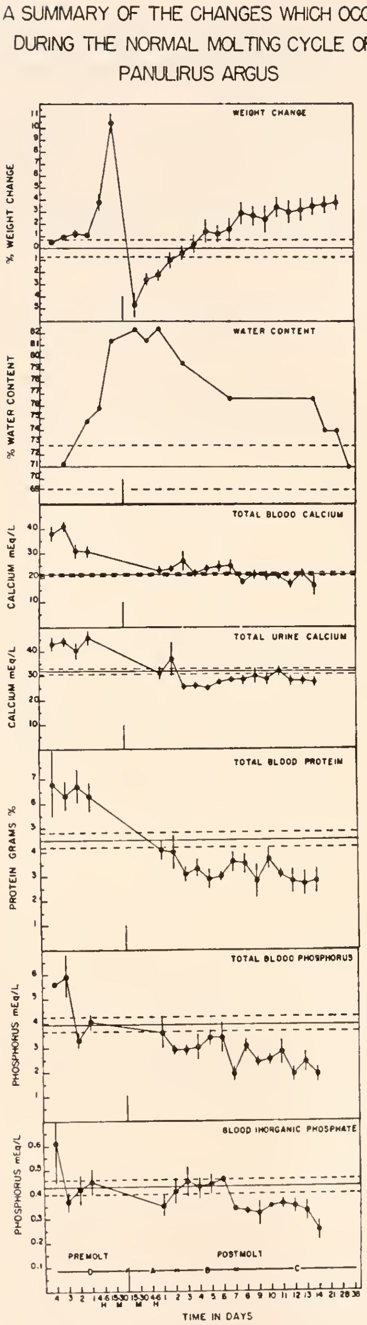

FIGURE 8. A summary of the changes which occur during the normal molting cycle. The graphs showing weight change and water content were taken from the paper of Travis, 1954. The vertical axis of the graph on water content, however, has been expanded to show more

count for most of the excess calcium which appears in the blood at this time. Some probably appears in very late Stage D, as a result of the increased uptake from sea water. However, since water is not taken in until one day preceding molt (Travis, 1954), it is evident that the maximum increases observed in *Panulirus* are not necessarily attributable to this source. Donahue (1953) observed that in *Homarus americanus* a rise in blood calcium begins ten hours preceding molt, and reaches a peak at two hours previous to molt. This rise would be well after most resorption had occurred from the old skeleton and would, in all probability, be due to the increased uptake of calcium from sea water absorbed shortly preceding molt.

Following molt, blood calcium in *Panulirus* approaches the normal intermolt (late Stage C) level rather rapidly and remains approximately normal for most of the fourteen day observation period (Stage A, B and early C) in spite of the amounts needed to calcify the new skeleton. Three factors appear to be responsible for this. The first factor is the concentration of calcium from the increased amount of sea water absorbed at and following molt (Travis, 1954). Maluf (1940) has shown that newly molted crayfish, *Cambarus clarkii,* will take up calcium ions at a greater rate than any other ions absorbed, though the hard shelled animals will not absorb any appreciable quantity of calcium. The second factor contributing to the relatively normal blood levels following molt is the reduced excretion of calcium. This is quite clear from Figure 4. Robertson (1937, 1939) has likewise shown that following molt, *Carcinus maenas* and *Cancer pagurus* excrete less calcium than they absorb. The third factor is the use of reserve calcium stored in the hepatopancreas and possibly other soft tissues preceding molt (Travis, 1955). The absorption cells of the hepatopancreas of *Panulirus* are filled with spherules of calcium phosphate—calcospherites—first observed in the hepatopancreas of *Carcinus maenas* by Robertson (1937). Thus, in spite of the fact that large amounts of calcium are needed for hardening of the new skeleton following molt, the animals are able to maintain a relatively normal blood level. Near the latter part of the fourteen-day observation period following molt (early Stage C) calcium falls slightly below the normal intermolt levels. It should be pointed out that in *Panulirus* the calcospherites in the absorbing cells of the hepatopancreas disappear simultaneously as calcification proceeds (to be discussed in a subsequent paper). Thus, the fall of blood calcium to slightly below normal levels is due to the depletion of the hepatopancreas reserves coupled with a decreased uptake of calcium from sea water. Similar variations observed in total blood calcium during the molting cycle have been shown in the fresh water crayfish, *Astacus fluviatilis* (Damboviceanu, 1930, 1932); the crab, *Maia squinado* (Drilhon, 1935); the marine isopod, *Ligia exotica* (Numanoi, 1937); and the crab, *Sesarma dehaani* (Numanoi, 1939). It will be noted from the foregoing list of animals that none, other than *Ligia,* is terrestrial. Because of the interesting, and indeed different, problems which confront terrestrial Crustacea, they offer a most interesting field for future investigation, particularly from the standpoint of mineral and water metabolism.

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clearly the changes in water content. Determinations of weight change and water content were made at 4–6 hours and 15–30 minutes preceding and following molt. This is not the case with blood and urine analyses.

Total urine calcium

Because of the amounts of calcium entering the blood from the old exoskeleton preceding molt (Stage D), large quantities are excreted at this time. On the third day before molt, when the blood calcium increases 18.4 mEq/L above the normal value of 22.8 mEq/L the amount of calcium excreted likewise rises to approximately 12.2 mEq/L above the normal intermolt value of 32.2 mEq/L. This would indicate that the increase of calcium in the hemolymph is largely, and quite effectively, controlled by the activity of the antennary or green glands. Robertson (1939) has similarly pointed out that prior to ecdysis in *Carcinus* and *Cancer* large amounts of calcium resorbed from the old skeleton pass into the surrounding sea water, this being controlled by the activity of the antennary glands.

Urine calcium approaches normal intermolt (late Stage C) values on the first day following molt (Stage A) and reaches a subnormal value by the third day. It might also be added that over-all hardening of the skeleton is obvious by the third day following molt (Stage B) but calcification actually begins on the second day following molt (to be discussed in a subsequent paper). The green glands are able to aid in conserving calcium for the hardening process after molt by excreting less (Fig. 4). Similar changes in urine calcium, namely, increased absorption and decreased excretion, have been noted by Robertson (1937) in *Carcinus* and *Cancer*. It might be pointed out in passing that inorganic phosphate could never be detected in the urine of *Panulirus* during any stage of the molting cycle. With these two ions, calcium and phosphorus, it is evident that the green glands of *Panulirus* are indeed tremendously important in the regulation of their concentrations within the blood. That the excretion of calcium is regulated in some manner so that a rather uniform concentration of calcium is maintained in the blood during most of the molting cycle, is further evidenced from the urine calcium to blood calcium ratios. The ratio of urine calcium to blood calcium in normal intermolt animals (late Stage C) is 1.4. Departure below this ratio occurs at four and three days preceding molt (late Stage D) and three through seven days following molt (Stage B), otherwise it remains relatively constant. Preceding molt, the departure may be explained by the fact that the large increase in blood calcium is simultaneously compensated for by an increased excretion of this ion. The levels of calcium in the blood and urine during the premolt period are approximately the same, the ratio being 1.1. From the third through the seventh day following molt the tendency to depart from the normal ratio is due to the fact that blood calcium remains relatively normal while urine calcium falls below normal. During the normal intermolt period (late Stage C) the ratio of urine calcium to blood calcium in *Panulirus* is very close to the ratio of 1.3 obtained by Bialaszwicz (1932) from *Maia*, a stenohaline poikilosmotic crab. Furthermore, Bialaszwicz showed that calcium, magnesium, and sulfate are actively excreted in proportions relatively greater than other ions such as chloride and potassum, thus giving somewhat higher urine than blood concentrations. These two stenohaline, poikilosmotic forms, *Maia* and *Panulirus*, live in an environment rich in salts and as a consequence normally maintain their blood isotonic with that of sea water by excreting an isotonic urine. In maintaining this isotonicity, however, certain ions are actively concentrated and conserved, such as phosphorus, while others may passively enter the body, such as calcium, but are selectively excreted. In the latter case fluctuations in the urine calcium to blood calcium ratios would be expected at various stages of the molting cycle. These fluctuations in the urine-to-blood ionic ratios are largely dependent upon the selective conservation or active excretion of certain ions.

Total blood protein

Total blood protein increases preceding molt, declines following molt, and reaches a subnormal value by the third day (Stage B). From this day throughout most of the fourteen-day postmolt observation period values remain below normal. Similar observations have been reported by Damboviceanu (1932) in *Astacus* and by Drilhon (1935) in *Maia*. Damboviceanu (1932) noted that the viscosity of the blood of *Astacus* increased just before and shortly following molt. If this viscosity increase were due to water leaving the blood and entering the tissues, thus concentrating the blood protein in the same given amount of fluid taken for analysis, one might suspect that an increase in protein preceding molt is only an apparent one. A viscosity increase in the blood, however, does not necessarily imply that loss of water is necessary for this change in the colloidal properties. This is markedly evident from the fact that Damboviceanu found the peak viscosity increase to occur shortly following molt, a time at which the blood protein levels are low due to dilution by the water absorbed at and following molt. He further showed that before the molt there are actual increases in various fractions of the total blood protein, that is, fibrogen and pseudoglobulin. Hemocyanin, however, showed little change preceding molt except at the beginning of gastrolith deposition, where his analyses indicate a decrease. The reason for the decrease in this fraction of total blood protein at this particular period of the molting cycle is unclear. The significant point, however, is that if increases in blood protein preceding molt were only apparent, due to loss of water from the blood, one would expect all fractions, including hemocyanin, to show an increase above normal. This is not the case. One might further expect that if water were leaving the blood and entering the tissues, the increase in osmotic pressure of the blood would facilitate water uptake. With regard to *Panulirus*, any changes in total water content do not begin until one day preceding molt (Fig. 8), that is, three days after a rise in protein is observed. The rise in blood protein of *Panulirus* observed four days through one day preceding molt would therefore appear to be a real one brought about by the large amount of organic material resorbed preceding molt (Table II). Since the crustacean skeleton contains both chitin and protein (Drach and Lafon, 1942; Lafon, 1948) resorption in the skeleton would mean that amino acids are pulled into the blood. These amino acids might, therefore, constitute an important stockpile for the resynthesis of new protein even though the animals do not feed (Stage D, A, and B). Some of the new protein is probably used in the formation of the pre- and even early post-exuvial layers of the skeleton.

Blood protein values decline rather rapidly and fall below normal shortly following molt. This fall, however, is largely caused by the dilution of the blood by water absorbed during and following molt (Fig. 8).

Total blood phosphorus

The elevation which occurs in total phosphorus at three days preceding molt is most likely accounted for by resorption from the old skeleton. It is pertinent to point out that total phosphorus determinations include not only inorganic but total acid-soluble and lipid phosphorus. Robertson (1937) has shown that 74% of the phosphorus withdrawn from the skeleton of *Carcinus* is organic. Most of this phosphorus is conveyed to the hepatopancreas and other soft tissues for storage. This was shown to be the case for *Cancer pagurus* (Paul and Sharpe, 1916), *Carcinus maenas* (Robertson, 1937), *Maia squinado* (Drach, 1939), and *Panulirus argus* (Travis, 1955).

As hardening of the new skeleton occurs, total phosphorus values decline and remain below normal during most of the fourteen-day observation period. This would appear to be caused by the dilution of the blood, during and after the molt, with phosphate-deficient sea water, coupled with depletion of phosphorus stores in the hepatopancreas and possibly other soft tissues. One might well expect this trend to occur since *Panulirus* does not feed two weeks before (Stage D) and one week following molt (Stage A and B). Phosphorus stores of the hepatopancreas quickly diminish as the new skeleton is progressively calcified during the early postmolt period (Robertson, 1937; Travis, 1955 and unpublished data). The blood concentration probably does not approach the normal value before the third week following molt. It is interesting to point out that Drilhon (1933, 1935) found that in *Maia* the total blood phosphorus remained constant throughout the pre- and postmolt period.

Blood inorganic phosphate

No marked change occurs in the concentration of blood inorganic phosphate of *Panulirus* preceding molt. Damboviceanu (1932) likewise found that there was no significant increase in inorganic phosphate of *Astacus* preceding molt. Drilhon (1933, 1935) found that in *Maia,* blood inorganic phosphate does not increase preceding molt but rises significantly in the act of molt (a period during which the present author did not run analyses) and falls sharply following molt. One might expect a slight rise preceding molt because of the amount of phosphorus resorbed from the old skeleton (Table II). This, however, was not the case in *Panulirus.* Although phosphate is withdrawn from the skeleton preceding molt, most of this, as Robertson (1937) has shown in *Carcinus,* is organic. Thus a rise in inorganic phosphorus of the blood might not be evident because of being combined as acid-soluble and/or lipid phosphorus and would therefore be reflected in total phosphorus determinations as indicated (Fig. 6). That blood and hepatopancreatic fatty acids and glycerides (to which phosphorus could be bound) increase preceding molt and slowly decline until feeding begins following molt (Stage C) has been shown by a number of investigators. Paul and Sharpe (1916) found that prior to molt, in *Cancer pagurus, Lithodes,* and *Homarus* there was increased fat storage (fatty acids and glycerides) in the hepatopancreas. Following molt, fatty acids and glycerides decreased in the hepatopancreas and increased in the blood. Damboviceanu (1932) demonstrated a rise in blood fatty acids of *Astacus* before molt, followed by a slow decline during the postmolt period. Drilhon (1935) showed an augmentation before molt of total lipids in the blood, hepatopancreas, and in genital organs of *Maia squinado.* This rise was followed by a fall during and following molt. Renaud (1949) found that in *Cancer pagurus* total lipids, fatty acids, phosphatides, cholesterol and total unsaponifiable fat increase to a peak in the hepatopancreas preceding molt, slowly fall following molt and reach their lowest point as the skeleton is hardened (Stage C). It is therefore quite apparent that the phosphate withdrawn from the old skeleton preceding molt could exist in the blood as acid-soluble or lipid phosphorus and would not appear as inorganic phosphorus. Prior to molt, therefore, the rise of blood fatty acids and glycerols could not only serve the function of combining with phosphorus withdrawn from the skeleton but could serve as carriers to convey phosphorus, via the blood, to the hepatopancreas for storage as calcospherites (Travis, 1955) preceding molt. They would likewise serve, in all probability, as carriers of phosphorus from the hepatopancreas to the integumental tissues as calcification begins following molt.

Blood inorganic phosphate concentrations of *Panulirus* fall significantly below normal at seven days following molt (end of Stage B) and remain low throughout the fourteen-day period of observation. Since the animals have gone through a previous period of inanition (two weeks preceding molt and one week following molt) one would expect a decline in inorganic phosphate as hardening of the skeleton proceeds. As this occurs, the stores of phosphorus in the hepatopancreas are drawn upon and soon depleted by the end of Stage B (Travis, 1955 and unpublished data). Blood levels fall concomitantly and probably do not approach normal until the phosphorus is replenished from food and until hardening of the skeleton is approximately complete.

SUMMARY

1. Total resorption of organic and mineral material in the area of softening is 19.7%. Of the total organic material, 23.0% is resorbed and of the total mineral material 12.8% is resorbed. The most abundant mineral element making up the exoskeleton of *Panulirus* is calcium, followed by magnesium and phosphorus (Table I). Of the total of each mineral element determined as the oxide, 8.4% CaO, 20.0% MgO, and 14.3% of P_2O_5 are resorbed.

2. The approximate amounts of organic and mineral material calculated, which would be resorbed from a carapace, devoid of endoskeleton and appendages, indicate that large quantities of these constituents would pass through the blood.

3. The Bermuda sea water is rich in calcium and represents the most important source of this element for *Panulirus argus*. The concentration of phosphorus, on the other hand, is very low. The food is probably the major source of this element.

4. Blood calcium rises markedly preceding molt, approaches the normal level rather rapidly following molt and remains approximately normal for most of the fourteen-day postmolt observation period (Stage A, B and early C). The large amounts of calcium resorbed from the old skeleton preceding molt account for most of the excess calcium which appears in the blood. Some probably appears as a result of increased uptake from sea water at and shortly following molt. In spite of the amount needed to calcify the new skeleton, blood levels remain relatively normal following molt. This is facilitated by concentration of calcium from the sea water absorbed at and following molt, by reduced excretion of this ion, and by the use of reserve calcium stored in the hepatopancreas as calcospherite.

5. Because of the amounts of calcium entering the blood from the old skeleton, large quantities are excreted during the premolt period. By the third day following molt (Stage B), urine calcium falls below normal intermolt levels (late Stage

C), a time at which over-all hardening of the skeleton has begun. The green glands, therefore, are able to aid in conserving calcium for the hardening process following molt by excreting less. No inorganic phosphate was ever detected in the urine during any stage of the molting cycle. The green glands of *Panulirus* are indeed important in the regulation of these two ions. The ratio of urine calcium to blood calcium in normal intermolt animals is 1.4. Departure downward from this ratio occurs four and three days preceding molt (late Stage D) and three through seven days following molt (Stage B), otherwise it remains relatively constant.

6. Blood protein of *Panulirus* increases preceding molt, declines following molt and reaches a subnormal value by the third day. The rise in blood protein preceding molt would appear to be brought about by the tremendous amount of organic material, some of which is amino acids, resorbed during this period. Synthesis of protein, by the reconversion of amino acids into new protein, could occur during this period even though the animals do not feed. The decline in blood protein below the normal intermolt level following molt is largely due to the dilution of blood proteins by the amount of water absorbed at and following molt.

7. The elevation which occurs in total phosphorus at three days preceding molt is most likely accounted for by resorption from the old skeleton (see Discussion). As hardening of the new skeleton occurs, total phosphorus values decline and remain below normal during most of the fourteen-day observation period. This would appear to be caused by dilution of the blood, at and following molt, with phosphate-deficient sea water, coupled with depletion of phosphorus stores in the hepatopancreas and possibly in other soft tissues.

8. No marked change occurs in blood inorganic phosphate preceding molt. This is probably due to the binding of the inorganic phosphate resorbed preceding molt, as acid-soluble or lipid phosphorus, thus causing increases in the total phosphorus content but not the inorganic fraction. Blood inorganic phosphate concentrations fall significantly below normal at seven days following molt (end of Stage B) and remain low throughout the fourteen-day period of observation. This is due to the fact that the animals have gone through a period of inanition (two weeks preceding molt and one week following molt) coupled with depletion of phosphorus stores in the hepatopancreas (end of Stage B) as hardening of the skeleton proceeds. Blood levels fall concomitantly with depletion of phosphorus stores and probably do not approach normal until phosphorus is replenished from food and until hardening of the skeleton is approximately complete.

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